Ripening and Abscission in Fruit of the Oil Palm (*Elaeis guineensis*, Jacq.): A Biochemical Investigation

Thesis

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Ripening and Abscission in Fruit of the Oil Palm (Elaeis guineensis, Jacq.) - A Biochemical Investigation

by

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ABSTRACT

Palm oil and palm kernel oil are two of the seventeen major oils and fats produced and traded in the world. The maximum harvestable yield of this plantation crop is, therefore, of major economic importance. Relevant aspects of the physiology and biochemistry of fruit ripening and the subsequent abscission of the ripe fruit has been the subject of this investigation.

The following major findings have shown that :-

The plant hormone ethylene is undetectable during the ripening period of about 25-40 days and the ripening of the fruit is not inducible by ethylene. The fruit does not soften and the pectic enzymes normally associated with cell wall changes in the ripening of other fruit are not induced. A cellulase (endo-ß-1,4-glucanhydrolase) is newly expressed in the fruit mesocarp and its activity increases in concert with ethylene production when at full ripeness but other pectolytic and cellulytic activities remain low.

The fruit abscission zone is a specialised tissue differentiated even at the pre-anthesis stage and is functional throughout development. It is rich in pectin, much of which appears to be polygalacturonate. The vascular tissue which passes through the zone is highly attenuated. Just prior to abscission, the barely detectable levels of ethylene rise manyfold, whereupon the fully ripe fruit is shed in less than 24 hours. A specific zone cellulase and exo- and endo-polygalacturonase isoenzymes are induced at this time with a substantial increase in the activity of other cell wall hydrolases such as ß-galactosidase and pectin methylesterase.

Non-abscinding mutant palms were discovered and the ethylene production and cell wall hydrolase activities in these were compared with normal fruit. Whilst ethylene production and cellulase activity in the fruit mesocarp occurred as in normal fruit, these activities do not lead to immediate abscission. After harvesting, cell separation at the abscission zone does eventually occur in bunches or spikelets of mutant fruit but only after a delay of several days. The cellulase induced in the zone is different from that in the normal and possesses many of the characteristics of mesocarp cellulase. In contrast, polygalacturonase isoenzymes show no differences from normal separated fruit and reach similar levels of activity whilst the activity of pectin methylesterase is substantially lower. Pectin metabolism in the mutant zone appears to be different from that of normal zones; there is preferential adsorption of the pI 6.2 polygalacturonase isoenzyme to the pectins and ^{13}C CP-MAS NMR spectroscopy suggested that the pectin is more methylated in the mutant. These results indicate that the zone-specific cellulase of the normal fruit is critical to the subsequent cell separation events and that zones of mutant fruit that do not produce this cellulase either rot on the palm or, as a post-harvest event, undergo cell separation and abscission by a variant cohort of enzymes.

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There is great scope for further work...

...Nothing in science ever seems to be finally settled.

ABBREVIATIONS

Abs$^-$	the non-abscinding leaf-petiole abscission zone of the lupin
ACC	1-aminocyclopropane-1-carboxylic acid
AOA	aminooxyacetic acid
APMSF	4-amidinophenylmethane sulphonyl fluoride
AVG	aminoethoxyvinyl glycine
ß-1,3-GH	ß-1,3-glucanhydrolase
cDNA	copy DNA
C_2H_4	ethylene
CMC	carboxymethyl cellulose
daa	days after anthesis
DI	deionised water
DM	degree of methylation
FFA	free fatty acid
FZ	fruit side of abscission zone after fruit separation
HM	high methylation
IAA	indole-3-acetic acid
IEF	isoelectric focusing
kD	kilodalton
Klu Mt	Kluang non-abscinding mutant palm
Lam	laminarinase
LM	low methylation
mRNA	messenger RNA
MW	molecular weight
MWCO	molecular weight cut-off
NS	not separated = unseparated abscission zone
OPA	*o*-phthaladehyde
P1, P2/3, P4/5	position of separation at oil palm abscission zone
PG	polygalacturonase
PGA	α-(1,4)-polygalacturonic acid
pI	isolectric point
PME	pectin methylesterase
PVP	polyvinyl polypyrollidone
PZ	pedicel side of abscission zone after fruit separation
RA	rudimentary androecium
TAG	triacylglycerol
TB1; TB2	tepal base 1 or 2
TTC	triphenyltetrazolium chloride
WT	wild-type

All abbreviations and prefixes of units of measure in this thesis are the Système Internationale d'Unités (SI), except volume and molar concentration (litres instead of dm^3; M instead of mol l^{-1}).

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Fig. i: **The Oil Palm, *Elaeis guineensis*, Jacq.**
An oil palm with unripe bunches of fruit (fruit appear black). The palms grow by about 0.3m per year and the height to which the trees grow eventually make them very difficult to harvest. The leaves (fronds) grow to between 8m and 10m long.

A

B

Fig. ii: A. Ripe harvested bunches. Each bunch can weigh up to 25kg on mature palms and they consist of many smaller spikelets (100-200 per bunch) of about 10-20 fruit, which become orange-red on ripening.

B. Individual spikelet, detached ripe fruits, and longitudinal section of fruits show the ripe orange mesocarp and white kernel flesh in the central nut.

PREFACE

THE ORIGIN AND DEVELOPMENT OF THE OIL PALM INDUSTRY

The appearance (Fig. i) and the economic value of the oil palm is still relatively unknown to many as we near the end of the twentieth century. This is in stark contrast to the coconut palm, a close relative of the oil palm, probably because the coconut has tasty, edible flesh - the coconut shies and the useful coconut-fibre doormats no doubt also have increased its familiarity! However, the oil palm has the distinction of contributing, in no small way, to the History of Science and the development of important industries.

The oil palm appears to have been indigenous to West and Central Africa. Its botanical classification, *Elaeis guineensis*, Jacq., is derived from the Greek *elaion* (oil) and the specific name of guineensis is indicative of its origin from the Guinea coast, West Africa. In 1763, Jacquin gave one of the earliest coloured illustrations of the oil palm tree and he has been memorialised in its scientific name (Hartley, 1988).

Due to the great technological advances of this century, more and more uses have been found for the oil obtained from the fruit of the oil palm. An unusual feature of this fruit is that it produces two vegetable oils - palm oil and palm-kernel oil. The former comes from the flesh of the fruit (the mesocarp) and the latter from the nut or kernel (Fig. ii). The extractable palm oil constitutes approximately 20% of the fruit's total weight and the palm kernel oil another 5%. When properly cultivated, the oil palm produces higher yields per hectare than any other oil-seed crop. Also, since the oil palm has a life-span for commercial purposes of about 25 years, productivity is combined with a perennial oil source, unlike many seed oils which are annual crops (Unilever plc, 1988).

These factors have no doubt contributed to the increased cultivation of the oil palm, and to its spread from a tropical African environment to similar environments around the world, including Malaysia, Indonesia, India, the West Indies, South and Central America. The Far East is now the world's major producer and the industry is expanding fast in tropical America. In 1994/5, the world production of palm oil and palm-kernel oil together was 23% of the total vegetable oils produced, and second only to the world production of soyabean oil (27% of the total vegetable oils) (Oil World Annual, 1996).

African carrying a harvested oil palm fruit bunch
(From: *Bulletin Agricole du Congo Belge*; Vol.38, 1947)

OTHER NIGERIAN SOUPS

ALAPA

This can be called palm oil soup, as distinct from palmnut soup, the preparation of which is described below

For *alapa* soup you need only meat, palm oil, pepper, onions, and tomatoes, but no green vegetables.

Alapa soup is often taken along with another green vegetable soup, e.g. plain *ewedu* soup.

METHOD
1. Boil the prepared meat; fry if desired.
2. Heat palm oil until it is hot but not bleached.
3. Add ground pepper, onions and tomatoes to the hot oil, and leave these ingredients in the oil to cook gently.
4. Before it is thoroughly cooked add a small quantity of water and let the ingredients and the water boil together for about 5 minutes then add ground tomatoes or tomato puree.
5. Finally, add the fried pieces of meat, season to taste and leave to simmer until well cooked.

"ALAPA" made from the orange mesocarp flesh

"BANGA" made from the white kernel flesh

BANGA OR PALM NUT SOUP (Obe eyin)

Ripe palm nuts
Pepper, **Onion**, **Tomatoes** — to taste
Locust beans (optional)
225g meat
Dried fish
Salt to taste

METHOD
1. Wash the palm nuts, put in a saucepan of water and bring to the boil.
2. Boil until cooked and the fibres soft.
3. Remove, put in a mortar and beat well to remove the husk containing the oil from the palm kernels; but be careful not to break the kernels.
4. Add warm water to the hot palm husks and mix well.
5. Sieve. The liquid collected contains the oil.
6. Put this liquid in a saucepan and heat, then add pepper, tomatoes, onions, locust beans, etc. Add dried fish or boiled meat as desired and cook as for stew.
7. Season well. Leave to simmer until cooked. Serve with yam foofoo or *eba*.

Fig. iii: **Traditional use of the oil palm in West Africa**
(Recipes from a Nigerian cookery book)

• The Oil Palm in its African Setting and its Discovery by Europeans

For centuries the oil palm remained a domestic plant to the Africans. However, the oil palm groves supplied more than just the need for oil and vitamin A in the diet. Although the fruit would be pounded, macerated and boiled by the African women to extract the oil for cooking (Fig. iii), the oil was also used as fuel for lamps, medicinally as a soothing ointment, and the hard shells of the inner kernel would serve as fuel for the fire. The palm leaves became roof-thatching, fencing, mats or brooms, and fibres from the leaf stalks could be twisted into ropes, then woven into baskets and fish traps. Sap drained from the immature male flower stalk made a refreshing drink and on fermentation was capable of yielding palm wine, vinegar and a strong alcoholic drink not unlike gin. In addition, as early as 1589, James Welsh, an English captain and trader, found in Benin a "good store" of soap smelling like "beaten violets" made from palm oil (probably the bleached palm oil perfumed by oxidised carotenoids). (Raymond, 1961; Opeke, 1982; Martin, 1988; Hartley, 1988).

During the sixteenth century, it was the slave trade which brought Europeans to the shores of West Africa and although palm oil is mentioned as food for the slaves, there was virtually no commerce other than the trade in slaves between 1562 and 1807. Even though James Welsh had brought back to England 32 barrels of palm oil in 1590, the trade did not grow (Stilliard, 1938). After 1807 the slave trade was suppressed by the British and substitute commodity trades from the West African coasts were sought by British merchants. Thus, palm oil acquired the unique distinction of being the major export commodity to Britain which replaced its trade in slaves (Hartley, 1988). In retrospect, it was said that "*the sneered at 'palm oil-ruffians' of the first half of this century, unconsciously, did more than anyone else to abolish the slave trade*" (Johnston, 1896). The prohibition to participate in the slave trade along with the end of the Napoleonic wars meant that British ships were freed for the trade in palm oil. Also, the slave trade had created the economic conditions in West Africa which enabled the oil palm farmers to respond rapidly to external demand (Northrup, 1976). The palm oil trade, according to Wilson (1852), was "*peculiarly a national one.....carried on in British vessels.....it is a barter trade, the palm oil being almost entirely paid for in the manufactures of Manchester, Birmingham and Glasgow*". In turn, the expanding demand for palm oil in Britain was due to important scientific advances which had brought about the "new vogues" of the nineteenth century.

The Contribution of the Oil Palm to the History of Science and the Development of Important Industries in the Nineteenth Century

It was fortuitous that during the last half of the eighteenth century, scientific investigation and invention were fuelling the beginnings of the Industrial Revolution (Trevelyan, 1964). Whilst the new trade in palm oil found a ready market in the soap-makers' boilers, even this trade would not have flourished had it not been for important discoveries made at about the same time.

In 1791 Nicholas Leblanc showed that alkali (sodium carbonate) could be obtained from common salt (brine). Then in the early 1800s the researches of Michel Eugène Chevreul revealed that oils and fats were compounds of fatty acids and glycerine, and in the process of saponification the fatty acids combined with the alkali to form sodium salts, possessing the lathering and cleansing properties of soap (Partington, 1964). Large scale manufacturing operations developed and the industry rapidly became able to process individual kettles of soap containing from 100,000 lbs to 1,000,000 lbs per charge (Anon, 1961a). The soap industry was thus revolutionised in the first half of the nineteenth century and, as a consequence of urban industrial life, the smoke and grime made soap a necessity where it had previously been a luxury. It is not surprising, therefore, that whilst the first record of the import of palm oil in Britain in 1790 was less than 130 tons, by the mid-1850s exports from West Africa had risen to around 25,000 tons per annum, and a decade later it was reported to total 40-42,000 tons per annum (Hartley, 1988; Northrup, 1976).

The increasing commercial importance of the fats and oils used in soap-making resulted in scientific analysis of their components. In 1813, Chevreul - the pioneer of fats and oils chemistry - discovered a fatty acid which he called "margarine" because its potash salt had a pearly appearance. (Greek: *margarites* - a pearl.) Palm oil was thought to contain margaric and oleic acids but later scientific analyses revealed that "margarine" was an impure mixture of two fatty acids - stearic acid and a "new" acid. In about 1840, according to Stenhouse (1841), a French scientist by the name of Edmond Frémy discovered this new acid when he examined the composition of palm oil and he gave it the name of "acide palmitique" (Dehérain, 1894). Since palm oil was very important to the British soap industry, John Stenhouse confirmed Frémy's experiments and reaffirmed the name of palmitic acid for the C_{16} fatty acid so abundant in palm oil (Stenhouse, 1841).

In this way, then, the Oil Palm has contributed to the fascinating History of Science - its legacy is contained in the non-systematic or "trivial" nomenclature of the organic (fatty) acids from butyric (C_4, Latin: *butyrum* - butter) to stearic (C_{18}, Greek: *stear* - suet/sheep fat) and, of course, palmitic after the oil palm itself. This terminology is still used by biochemists today and it reflects the biological material analysed by these nineteenth century chemists (Partington, 1964).

Advances other than in the soap industry which further boosted the demand for palm oil included the stearic or stearin candle. Tallow (beef suet) candles were in common use early in the nineteenth century. They were soft, needed constant attention and their smoky flames gave off unpleasant odours. Chevreul had discovered that the solid fatty acids obtainable from tallow could be made into better candles which burnt with a clear flame. In 1825 Chevreul and Gay-Lussac obtained patents in France and England for making candles from "stearin" (this was a mixture of fatty acids - mainly palmitic and stearic) but they failed to produce candles at an economic price. Others soon succeeded by saponifying the fat with lime, rather than the more costly soda or potash, and this resulted in the widespread manufacture of the "stearic" candle (Lemay & Oesper, 1948; Smeaton, 1989). Palm oil was now about to claim a position of great importance in this industry. A London firm, Price's Patent Candle Company, patented their process for a treatment of fatty acids and, as a result, palm oil was used in enormous quantities for the production of palmitic acid at their candle works and by other manufacturers in Great Britain (Wilson, 1852). The "Price's Candle Stove" was developed for "*urgent warmth and cooking power*" and was used by the soldiers who fought at the Crimea (Wilson, 1856). In addition, the improvements in candle manufacture inaugurated a new era in lighting making possible "night lights". In his lecture "On the Stearic Candle Manufacture" to the Society of Arts in 1852, Wilson said that "*palm oil has become one of the principal raw materials used in the manufacture of stearic candles*".

Large quantities of palm oil were consumed both by the railway companies (who used it almost exclusively for greasing axle boxes of railway carriages) and in the tin-plate industry. The use of tin-plated iron became a major industry as containers of tin-plate began to displace the more fragile glass and pottery (Tylecote, 1992). Spoons of tinned iron were said to be intermediate in social order between the silverware of the rich and the wooden spoons of the poor! In the late eighteenth century there was also a growing interest in the preservation of food and in 1787 the Society of Arts offered a prize for a

method of preservation which would retain the fresh flavour of foodstuffs. This new food canning industry which developed in the nineteenth century provided the main stimulus for the expansion of tin-plate production (Minchinton, 1957a). Palm oil was initially used to prevent oxidation of the iron and as a flux before tinning. By the end of the nineteenth century steel began to replace iron and there was increased mechanisation of the tinning process but palm oil did not become redundant. The freshly tin-plated steel was now passed through a large "grease-pot" of hot palm oil. This helped to remove the excess tin, distribute the remainder evenly over the surface and prevent oxidation of the tin-plate as it cooled (Minchinton, 1957b; Anon, 1961b). Although electrolytic tin-plating has replaced this "hot dip" method, palm oil is still well known to many metallurgists as one of the first "hot rolling oils".

Palm oil found a use in the manufacture of a "liquid artificial ether" which gave aroma to wines, and the saponified oil was a source of glycerine. Price's Patent Candle Company recognised the value of glycerine and in 1854 patented a process for the production of purified glycerine from palm oil (Wilson, 1856). Glycerine was used in numerous medical applications, in photography to preserve film, in the manufacture of colours, as a cosmetic and in perfumery. It also played an important part in the History of Science. Glycerine embalmed and preserved the colour and odour of objects of natural history. Thus, it was suggested that specimens of scientific interest, such as "*the brilliantly-tinted fishes of the Coral Islands and tropical coasts*", discovered by nineteenth century naturalists "*might be brought back to England in kegs of glycerine*" (Wilson, 1856). The preservation of these specimens in their natural state, despite many months of travelling, was no doubt a stimulus to their further study. This glycerine was derived mainly from palm oil. Later, glycerine came to be in great demand for the manufacture of dynamite and high explosives used in the engineering work of the Panama Canal and on the land in South Africa. In their book, Palm Oil & Kernels - The Consorts of the West Coast, Billows & Beckwith (1913) note "*Palm oil may be said to have almost innumerable uses. Even leakage from the casks in the ships' hold during transit is carefully scraped up and sold for the manufacture of metal polish*" ! About this time, economical methods for cracking the palm-kernel shells were developed and palm-kernel oil, as well as palm oil, was then used in the manufacture of candles, lubricants and soap. The resulting palm-kernel cake that remained after expression of the oil was sold as cattle feed (Billows & Beckwith, 1913).

However, probably the most important development was promoted by Napoleon III. He encouraged French chemists to prepare a product that could take the place of butter but would be cheaper and have better keeping properties. The working-man's diet remained short of fat because butter was not only expensive but supplies were not sufficient to reach this rapidly increasing population. In 1869 a French chemist, Hippolyte Mège-Mouriès, theorised that since fasting cows produced milk from which it is impossible to make butter, the butterfat itself must be derived from beef suet and not from the milk. He developed a rather complicated process of pressing beef suet at 30°-40°C to finally obtain a fat that melted at 20°-25°C - it was, in the terminology of the day, "oleomargarine". (By this time, margarine was the name given to impure tripalmitin and impure oleopalmitin was called oleomargarine.) Mège's product was a household fat which could replace various other fats, even butter, for cooking purposes and in 1872, it was put on sale in Paris as "margarine" (Wilson, 1954; van Stuyvenberg, 1969). Others saw the great potential of his process which, shorn of its superfluous trimmings and adapted to large-scale production, was to supply a butter substitute to the working-class masses. Initially, animal fats provided the raw materials for the "margarine", so named by a British Act of Parliament in 1887, but newly developed refining and hydrogenating processes enabled manufacturers to use vegetable oils including palm oil and palm-kernel oil. These oils, well established in other industries, had now entered the Food Industry and would prove to be a most versatile commodity. Even the edible oil from the palm kernel was soon employed in the manufacture of chocolate since it was found that it could replace, in part, the expensive cocoa butter and thus reduce costs (Billows & Beckwith, 1913).

The situation then, at the end of the nineteenth and early years of the twentieth centuries, was that the oil from the West African Oil Palm had become very well known to commerce and industry of the day. In fact, the reputation of the palm oil trade was such that jocular allusion may have been made in this pun referring to bribery: "*A judicious application of palm-oil will always produce temporary blindness in the officials on duty*"! (King, 1896).

- **The Oil Palm Industry Today**

In order to supply the demand for palm oil and palm-kernel oil, there would no longer be a sole dependence on the natural oil palm groves of West and Central Africa.

Surprisingly, commercial development in the Far East was more successful than in Africa, the home of the oil palm. Four trees were received by the Buitenzorg Botanical Gardens in Java around 1848. These four trees provided seed for all the oil palms in the Far East and they grew as well, if not better, than in Africa. Commercial development was undertaken by a Belgian firm in 1911, and large plantations developed in Sumatra. Also, seed from these plantations was used in the large-scale planting of the oil palm in Malaya in 1917. In Nigeria, the Cameroons and the Congo, European interest in the commercial development of plantations began from around 1910 onwards. Sir William Lever was concerned with the growing demand for palm oil, especially by the margarine makers, and was convinced that this was driving up the prices. He hoped that, by increasing world supplies of raw materials, it would bring prices down. At first, five factories were erected at various places in the Congo, which processed fruit purchased from the African, but from about 1935 the Unilever concern fostered the development of its own plantations there (Wilson, 1954).

It was also the Unilever company which initiated research into the clonal propagation of the oil palm by tissue culture. In other tree crops, such as rubber and cocoa, it is possible to by-pass propagation by seed and so limit the genetic variation, thus clonal plants can be produced from the best trees by taking cuttings or by grafting. With oil palm conventional methods of vegetative propagation could not be used but eventually the tissue culture technique allowed the same ends to be achieved. Research from the late 1960s onwards resulted in the production of elite, top quality, clonal palms which proved to be more resistant to disease, matured earlier, were higher yielding by 25-30% and produced fruit at low level. Mature palms from clones, such as 90A, 926, 975, 476G, 271D and 115E (Fig. i shows clone 115E), are being cultivated on Unilever plantations in Malaysia today and these clones, especially 90A, 926 and 271D, have supplied the fruit used in the present study.

During the last 50 years the oil palm industry has successfully expanded and grown due to the development of efficient plantation management, selection of elite high-yielding palms for breeding and cloning, and improved processing and marketing of palm and palm kernel oil. It was recognised at an early stage that when the fruit were ripe and loose on the bunch, and especially after bunches were harvested, the mesocarp began to hydrolyse its triglycerides which increased the free fatty acid (FFA) content of the oil (Vanneck, 1947). In former times, the main outlets for palm oil were to the soap, candle and tin-

plate industries - these industries being the least exacting in their requirement for oil with a very low FFA content. It was not uncommon for palm oil to have up to 50% or more FFA content (Raymond, 1961; Coursey, 1963) and this was not satisfactory for use in food products such as margarine. Nowadays, palm oil is used mainly in food applications and technological advances have been employed to produce palm oil with an FFA content of 2-3% or less (Wood & Beattie, 1981). On the plantations, ripe fruit bunches are monitored for the fall of the first few loose fruits per bunch, and this criterion is used to determine the time to harvest for the maximum amount of oil. The cut bunches, which can weigh over 25 kilograms on mature palms, are immediately collected by estate truck and speedily delivered to the factory mill located on the plantation site. Here the fruit are loaded into large pressure vessels/cages and steam-heat sterilised. This stabilises the oil quality in terms of the development of FFA. The fruits are then stripped from the bunches, pressed for extraction of the crude palm oil which is clarified from the unwanted water, solid fruit particles and other impurities. The kernels are recovered, dried and cracked, and the kernel flesh is then pressed for the extraction of the palm kernel oil. The remaining kernel cake is sold for livestock feed and the bunch and fruit fibre with kernel-shell residues can be used to fire the boilers which generate steam and electricity for the factory. Slow incineration of bunch fibre gives an ash rich in potassium which can be used as a fertiliser and, recently, the feasibility of using the ash as a cement replacement material has been studied (Tay & Show, 1995). By means of the appropriate technology, the trunks, fronds and kernel shells can be utilised for the production of furniture, building materials (such as wood-panel and board), pulp and paper. Nothing is wasted.

The crude palm oil from the mill is transported to refineries and further processed to produce a neutral near-white oil after removal of free fatty acids, colour and unwanted flavour. Until relatively recently, the crude palm oil was then only separated into its liquid "palm olein" (the more unsaturated and homogeneous triacylglycerol fraction) and solid "palm stearin" (the more saturated triacylglycerol fraction), but the current trend of refining is in the downstream processing of specialty fats and oleochemicals. Fractionation, hydrogenation, interesterification and derivatisation can modify both palm and palm-kernel oils making them suitable for a wide variety of products as shown in Table i and Figs. iv and v. An interesting example of the downstream processing of palm oil is "Betapol", a blend of triacylglycerols (TAG) prepared especially for the feeds of premature babies, and it is manufactured by interesterification techniques (Kavanagh,

Table i: PRODUCTS MADE FROM PALM OIL AND PALM KERNEL OIL

90% of all palm oil and palm kernel oil produced is used for food purposes, the remaining 10% goes into non-food applications. (Information in this table is compiled from publications by MPOPC and PORIM, 1996.)

PALM OIL

CRUDE PALM OIL

- Used directly as a fuel to run cars with suitable modified (Elsbett) engines
- Drilling mud made with palm oil for drilling through sensitive oil producing formations

REFINED, BLEACHED AND DEODORISED (RBD) PALM OIL

- Margarines
- Shortenings
- Vanaspati/vegetable ghee
- Frying fats
- Ice cream
- Salad dressings
- Dough fat
- Imitation cheese
- Instant noodles

FRACTIONATION OF RBD PALM OIL

- Olein, super olein) food machinery lubricating oils, pan greasing, textile oils
- Mid-fraction) textile oils, salad oil (olein), instant noodles (olein)
- Stearin) puff pastry shortening (stearin)

HYDROGENATION/INTERESTERIFICATION/BLENDING OF FRACTIONS

- Olein - cooking oil, butter fat replacer for ice-cream, toffee whiteners, cocoa butter extender/replacer in confectionery
- Mid-fraction - cocoa butter equivalents in confectionery
- Stearin - margarine, shortenings, "Betapol", peanut butter

EPOXIDATION OF FRACTIONS

- Plasticizers, stabilizers for plastics like PVC

METHYLESTERIFIED PALM OIL AND STEARIN

- Diesel fuel substitute

"SPLIT" PALM OIL

- Fatty acids - soap, candles, fatty alcohols for anionic and non-anionic surfactants, amines and amides for quaternary ammoniium compounds, Vitamin E tocopherols and trienols from palm fatty acid distillate
- Glycerol - food emulsifiers, humectants in cosmetics, explosives, as a plasticiser in polar polymers, as antifreeze, production of mono- and diglycerides, production of polyols, polyesters and polyurethanes, in medical applications such as nitroglycerine, moisturising creams, a freezing medium for living tissues, suppositories, as a vehicle for drugs applied to skin

PALM KERNEL OIL

REFINED, BLEACHED AND DEODORISED (RBD) PALM KERNEL OIL

- Medium chain triacylglycerols act as carriers for flavour agents as solvents for medicines, as lubricants and mould release agents in the food industry and in creams and lotions

FRACTIONATION OF RBD PALM KERNEL OIL

- Olein - margarines; hydrogenation - confectioneries, coffee whitener, filling fats, coating fats
- Stearin - toffee (hydrogenated PK stearin), cocoa butter substitute in confectionery

RE-ESTERIFICATION

- Medium chain triacylglycerols are prepared for those with nutritional disorders in fat absorption like cystic fibrosis (they are less hydrophobic and more easily absorbed in the intestine)

"SPLIT" PALM KERNEL OIL

- Fatty acids - C_{12}-C_{14} has good foaming properties and mildness, used for detergents/surfactants
- Glycerol - See above under "Split" Palm Oil

1995). Traditionally, blends of vegetable oils have been used to supply the fat content in infant formula feeds. However, human milk has TAG with 70% palmitic acid at the 2-position, whilst most vegetable TAGs do not. Palm oil contains the TAG - tripalmitin, so specific enzymes can modify the 1- and 3-positions in this TAG and exchange or interesterify the palmitic acid at these positions with unsaturated fatty acids producing a TAG very similar to human milk. (The 2-position or beta position in the tripalmitin of palm oil is unchanged, so that the trade name Betapol is an abbreviation for **Beta** palm **oil**.)

For many years palm oil in the diet was thought to present a particular health hazard because it contains a high saturated fatty acid content (palmitic: 45%). In fact, in the U.S.A. research reports suggested that the saturated fats of the so-called "Tropical Oil" (meaning palm and coconut oils) raised serum cholesterol levels and were atherogenic (O'Holohan, 1991). Reports like these were used in an anti-tropical oils campaign launched by the American Soyabean Association. For example, some products were even labelled "Contains no Palm Oil" (C.W.S. Hartley, personal communication, 1996) and this campaign was only called off in July 1989 (Lim, 1991). However, clinical and laboratory studies on the nutrition and health aspects of palm oil indicate that there appear to be positive health benefits. There is the absence of trans-fatty acids, along with the high Vitamin A (ß-carotene) and Vitamin E (tocotrienols) content (even after processing) which can act as antioxidant and anti-thrombotic agents. Additionally, palm oil itself has a lipid-lowering effect with no adverse effect on serum cholesterol; that is, it is safe in relation to atherosclerosis (Khor & Tan, 1991). On-going research by the Palm Oil Research Institute of Malaysia has shown that palm oil can be used in products as diverse as imitation palm-based cheese and "Pure Palm" hand and body lotion (Fig. v) to the use of palm-based methyl-esters as diesel fuel and epoxidised palm oil which produces a plasticiser and stabiliser for plastics such as PVC (MPOPC, 1996; PORIM, 1996).

It is clear from the foregoing that just as palm oil was said to have "*innumerable uses*" at the turn of this century (Billows & Beckwith, 1913) so as the century ends, the same situation prevails, and the Oil Palm, *Elaeis guineensis* can be said to be one of the very few plants with apparently limitless applications.

Fig. iv: Products prepared from or containing palm oil and available from local shops in the U.K.


INGREDIENTS
PALM OIL, RAPESEED OIL, SOYA OIL
100% FAT
FRYING
BAKING
ROASTING
SPREADING
London SE1 9LL


SAINSBURY'S
SOLID
VEGETABLE OIL
100% PURE VEGETABLE OIL
FOR DEEP
AND SHALLOW
FRYING


INGREDIENTS
POTATOES · PALM OIL · SALT
Elliott
35%
REDUCED FAT
CRINKLES
READY SALTED


The Village Bakery
MELMERBY
Organic bread and cakes made by hand, baked in wood-fired brick ovens.
HAZELNUT & FENNEL BREAD
A swirl of chestnut, fennel and ale pâté in a light brown spelt bread, studded with toasted hazelnuts
Ingredients: Spelt Flour*, Unbleached Wheat Flour*, Hazelnuts*, Chestnut Purée, Onions*, Barley Malt Extract*, Golden Promise Beer*, Sunflower Oil*, Free Range Eggs*, Stoneground Wholemeal Flour*, Yeast, Oranges*, Sea Salt, Fennel Seed*, Garlic*, Spices*, Palm Oil*


NO TRANS FATS
312 g
11 oz
Whole Earth
CRUNCHY
PEANUT
BUTTER
NO SUGAR ADDED
NO TRANS FATS
INGREDIENTS: FRESH ROASTED PEANUTS, PALM OIL(3%), SEASALT(1%).
Whole Earth Peanut Butter is made without sugar or hydrogenated fat.

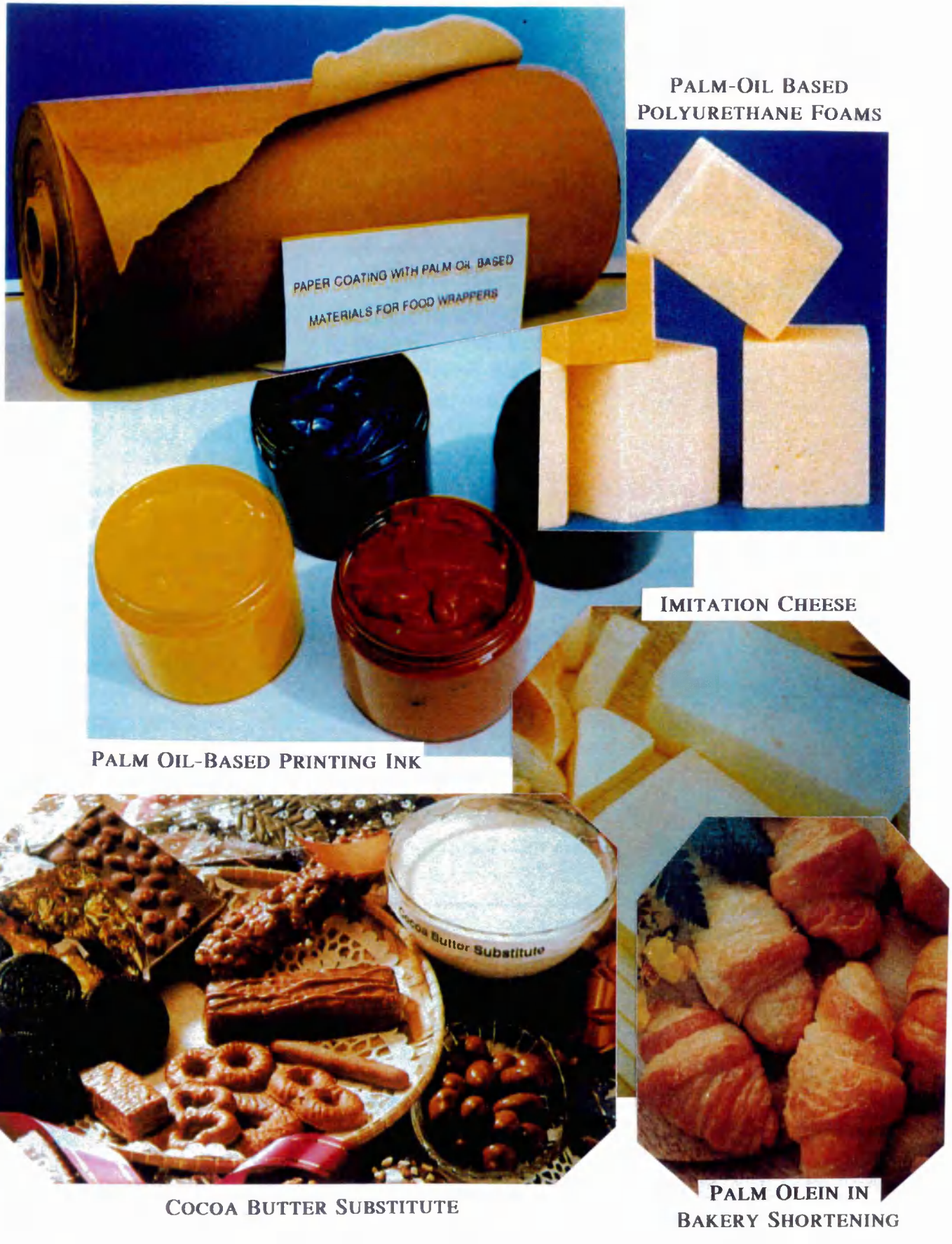


Fig. v: Some food and non-food uses of palm oil developed by PORIM in Malaysia (photographs of products courtesy of PORIM, Brickendonbury, Herts., and from the PORIM publications: *Palm Oil Developments*, Nos. 23, 24, 26; *Palm Oil Technical Bulletin*, May 1996 and Nov-Dec, 1997).


PALMVITEE
PENTING UNTUK
PURE PALM
PALMVITEE
PORIM
PORIM
NUTOLEIN
GOLDEN PALM OLEIN

CHAPTER 1
INTRODUCTION

1.1 THE BIOCHEMISTRY OF FRUIT RIPENING

The biochemistry of fruit ripening - which can be either the pre- or post-harvest metabolism - is a major area of study because it is of enormous commercial importance. Colour, texture, flavour and aroma are the ripening changes most readily recognised but these observable physical changes must be the result of a combination of altered metabolic control of existing pathways and new ripening-specific gene expressions. These major ripening changes have been reviewed in 1987 by both Brady, and Tucker & Grierson. Since that time, molecular biological techniques, for example, antisense RNA technology, and the genetic manipulation of naturally occurring ripening-impaired mutants have contributed to a greatly improved understanding of the biochemistry and regulation of fruit ripening (Gray *et al*, 1994; Grierson & Fray, 1994; Picton *et al*, 1995a).

The tomato has been the fruit most often used in these studies and over 25 ripening-related mRNAs have been cloned, several of which have been assigned precise functions (Picton *et al*, 1995a). Transgenic tomato plants expressing antisense RNA, which causes a reduction in the level of expression of the endogenous gene, has allowed examination of the role of specific genes in the ripening process. An additional tool is the many ripening mutants of tomato which have been described and can be similarly manipulated by insertion of known genes. McGlasson (1985) notes that these mutations have little or no effect on growth and development during pre-ripening stages but exert their effect during the period that normal fruit undergo ripening. Examples of some pleiotropic mutations (those which have many aspects of ripening perturbed) in tomato are: ripening inhibitor (*rin*), non-ripening (*nor*) and Neverripe (*Nr*) mutants. The complexity of fruit ripening is demonstrated in these tomato mutants by the fact that even though the genes of the mutations *rin*, *nor* and *Nr* are located on different chromosomes, they produce several similar responses. Thus, the tomato has become a "model" system to investigate developmentally regulated changes in gene expression, and its DNA libraries and cDNA probes have been used to identify specific ripening-related sequences in other plants such as the peach (Lee *et al*, 1990), and melon (Karvouni *et al*, 1995). Three important aspects of ripening have been investigated in these studies:

- The role of ethylene as the "ripening hormone" and its mechanism of action
- The enzymatic basis of softening and texture changes
- Carotenoid biosynthesis and its regulation

The effects of the various plant hormones, in particular the role of ethylene and signalling mechanisms in ripening, are discussed in Section 1.1.1, and greater detail is presented on the fruit cell wall with its associated enzymes and ripening-related texture changes in Section 1.1.2.

Studies on carotenoid biosynthesis are of interest since oil palm fruit also undergo the characteristic colour change from unripe fruit which are yellow-green with purple tops to fruit which are bright orange-red at full ripeness, indicative of increased carotenogenesis. Colour changes (other than accumulation of anthocyanins in the vacuole) are due to the loss of chlorophyll (and hence photosynthesis), and the accumulation and biosynthesis of carotenoids in the plastids (that is, the transformation of chloroplasts to chromoplasts). In tomato there is a specific ripening-induced pathway to lycopene formation. This occurs by induction of the gene for phytoene synthase (*PSY*1) by ethylene and is a different gene from the one expressed in vegetative tissues (*PSY*2) (Bartley *et al*, 1994). Pecker *et al* (1996) report down regulation of the gene for lycopene cyclase so that lycopene, rather than carotene, accumulates. However, in another solanaceous plant, pepper, the *PSY* mRNA levels are only slightly induced by ripening (Römer *et al*, 1993). This indicates that the regulation of the same gene may be quite different even in members of the same family. Bartley *et al* (1994) mentioned the research opportunities to manipulate the quantity and quality of carotenoids in food and feed crops. This may prove to be directly applicable to the oil palm since red palm oil is becoming more popular and the ß-carotene it contains appears to have health benefits as a precursor of vitamin A and for its anti-oxidant properties. Although changes in chlorophyll and carotenoid content of ripening palm fruit have been reported (Ikemefuna & Adamson, 1984) (Section 1.3.1), regulation of carotenogenesis in oil palm fruit has not as yet been investigated.

1.1.1 Plant Hormones and Putative Signalling Mechanisms in Fruit Ripening

Tucker & Grierson (1987) note that all the major phytohormones are probably involved during ripening but a complete understanding of the effects of different hormone levels and/or ratios is still unclear. The regulation of hormone or hormone receptor

concentration and/or the changes in sensitivity towards them modulate their action and the biochemical mechanisms controlling this are gradually being elucidated. However, although plant hormones themselves can delay and/or accelerate ripening, the mechanism for the initiation of ripening is unknown in both climacteric and non-climacteric fruits and, clearly, there are biochemical events which must precede and signal the induction of the specific hormones and/or receptors. The five principal classes of phytohormones are: gibberellins, cytokinins, abscisic acid, auxins and ethylene.

Gibberellins, Cytokinins and Abscisic Acid

The gibberellins and cytokinins have been shown to reduce or delay various aspects of ripening (Abeles *et al*, 1992), and this inhibition has been ascribed to their anti-senescence effects (Brady, 1987). Gibberellin (GA_3) treatment in tomato and sweet cherry delayed the red colour development, decreased polygalacturonase and Cx-cellulase activities and the fruit were more firm than controls (Andrews & Li, 1995). The same has been reported in fruit such as the tomato (Babbitt *et al*, 1973) and persimmon (Ben-Arie *et al*, 1995). Abscisic acid and its conjugates have been shown to rise late in development or during ripening (for example in plum, apricot and muskmelon) and treatment with ABA itself may advance ripening (Brady, 1987; Dunlap *et al*, 1996). In grape and peach increased sensitivity of tissues to applied ethylene correlated with an accumulation of abscisic acid in the developing fruit (Brady & Speirs, 1991). Abscisic acid frequently promotes the senescence of green tissues (Brady, 1987). THIS HAS BEEN OBSERVED IN OUR OWN EXPERIMENTS WITH THE OIL PALM. WHEN APPLIED TO THE UNRIPE SPIKELETS OF OIL PALM FRUIT, THE ABA-TREATED FRUIT WERE LESS GREEN THAN THE WATER CONTROLS AFTER FIVE DAYS, APPEARING "MORE RIPE".

Auxin

Auxin or indole-3-acetic acid (IAA) content in fruits appears to be related to ripening in different ways.

- IAA levels ***decrease***, ripening advances with ***no*** involvement of ***ethylene***.
  Lowered levels of auxin have been shown to advance ripening in strawberry, but ripening in this fruit appears to be independent of ethylene and applied ethylene does not advance ripening (Given *et al*, 1988; Brady & Speirs, 1991). The

achenes of strawberry are a rich source of IAA and when removed, there is accelerated accumulation of anthocyanin with subsequent loss of chlorophyll and firmness. Application of synthetic auxins, either through the peduncle or to the de-achened surface, delayed these ripening changes but phenoxyacetic acid, an inactive auxin analogue, did not delay ripening indicating this was an auxin-specific effect. The mRNA pattern of these changes has been analysed by Manning (1994). He reported that, again, removal of the achenes from a segment accelerated ripening producing a mRNA pattern similar to normal ripe fruit, whereas application of a synthetic auxin (10^{-5}M) to the de-achened receptacle produced a mRNA pattern similar to that in mature green fruit.

- IAA levels ***decrease***, and in mature green tissues ***ethylene*** synthesis ***increases***. Tomato IAA levels decrease following the early stages of fruit expansion, and in the mature-green fruit IAA levels are very low just before the burst of ethylene (Buta & Spaulding, 1994). The application of IAA (10^{-5}M) to an *in vitro* fruit culture (started from the immature flowers) at the mature green stage retarded its further ripening, increasing the time period between breaker and red-ripe stages (Cohen, 1996). Thus, it may be necessary for auxin levels to progressively decrease before ethylene is induced, and this also has been suggested for ripening pears (Frenkel & Dyck, 1973).

- IAA levels ***increase***, and in mature green tissues ***ethylene*** synthesis ***increases***. IAA levels have been reported to increase greatly in the period preceding the ethylene burst at ripening in apple and peach (Mousdale & Knee, 1982; Miller *et al*, 1987). In the fig, at the initiation of ripening, IAA levels increased about 2-fold for a short time (Abeles *et al*, 1992). These results may indicate that high IAA levels can induce ethylene production at ripening in some fruits (see **Ethylene** below).

Auxin metabolism involves formation of conjugates such as IAA-inositol, IAA-glucose, IAA-aspartate and others. Slovin & Cohen (1993) suggest that conjugation is a mechanism for fine regulation of auxin levels in cells. In this way a changing conjugation capacity during fruit development and ripening provides a mechanism whereby auxin exerts specific effects during defined stages of the ripening process.

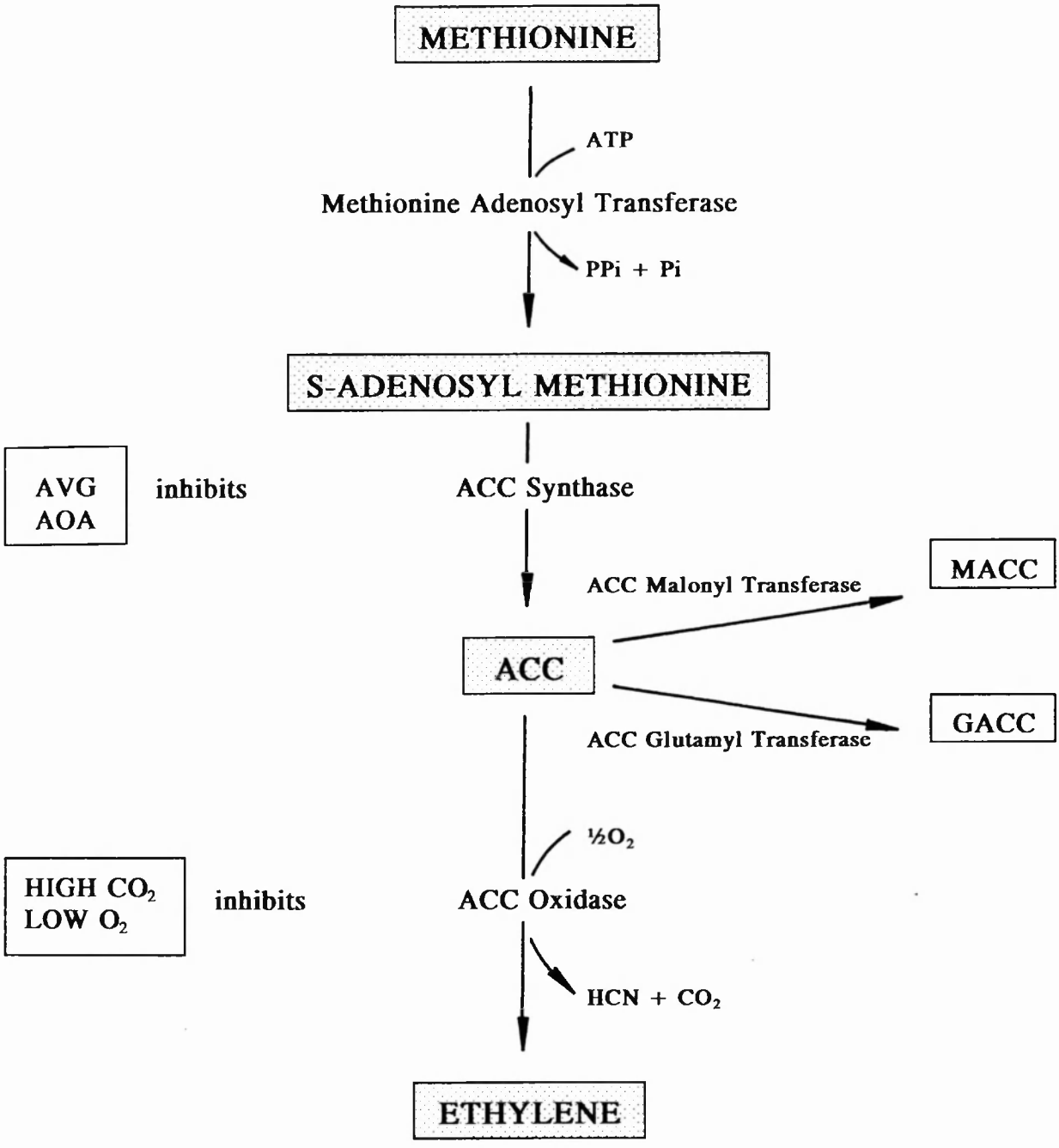


FIG. 1.1: PATHWAY OF ETHYLENE BIOSYNTHESIS AND CONJUGATION

Ripening Control: AOA or AVG inhibits ACC synthase
High CO_2 or low O_2 inhibits ACC oxidase

Ethylene

Enhancement of fruit ripening by ethylene is one of the most widely investigated and well established effects of this hormone. For many years ethylene gas was used in storage rooms to enhance the ripening of bananas but nowadays the same effect is achieved by sealing hands of bananas in plastic bags and storing in temperature controlled rooms; the temperature regime alone determines the onset of ethylene production (Lürssen, 1991).

The pathway of ethylene biosynthesis in higher plants was first elucidated in apple fruit tissue by Adams & Yang in 1979 (Fig. 1.1). Ethylene is synthesized from the amino acid methionine and the product S-adenosyl methionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by a multigene enzyme ACC synthase. This enzyme is inhibited by rhizobitoxine and its analogues, such as aminoethoxyvinyl glycine (AVG) and aminooxyacetic acid (AOA). When applied to preclimacteric apples and pears AVG delayed ripening (McGlasson, 1985). Another multigene enzyme ACC oxidase converts ACC to ethylene. Since this enzyme requires oxygen, knowledge of the antagonism between carbon dioxide and ethylene is used to control the ripening process in controlled-atmosphere fruit storage, for example, apple and avocado (Lürssen, 1991).

The pattern of respiration and ethylene synthesis in ripening fruit (***after harvesting***) has been broadly classified as either climacteric or non-climacteric. In **climacteric** fruit at an early stage of ripening when the fruit are mature but still green, such as the tomato, banana, peach and apple, there is a peak in respiration (this is referred to as the climacteric) and a concomitant burst of ethylene synthesis. In recent years, convincing evidence has accumulated to suggest that ethylene is a key regulatory molecule in the ripening of climacteric fruit and not a by-product of ripening (Oeller *et al*, 1991; Theologis, 1992). In fact, nowadays the terms "climacteric" and "non-climacteric" are used to describe fruit which ripen when treated with ethylene and those that do not (Abeles *et al*, 1992). **Non-climacteric** fruit such as the pepper, cherry, strawberry, and citrus (although according to Abeles *et al* (1992) some species of citrus are now considered climacteric) do not show any increase in respiration during ripening, even though they may produce low levels of ethylene (about 0.04 μl kg^{-1} h^{-1}) throughout this period (Tucker & Grierson, 1987).

The regulation of ethylene production in ripening fruit tissue is complex and studies have shown that ethylene can regulate its own biosynthesis. Methods of regulation, reviewed by Mattoo & White (1991) and Fluhr & Mattoo (1996), include:

- **Autocatalysis:** This occurs in climacteric fruit which are mature but still unripe or green. A massive increase in ethylene production is triggered by exposure to ethylene itself, and it appears that ethylene induces the enzymes of its own biosynthetic pathway. Initially, ACC oxidase activity increases in response to applied ethylene in normal mature green tomato fruit but, with a longer exposure, the fruit shows increases in ACC levels and endogenous ethylene production, indicating induction of ACC synthase.

- **Conjugation:** ACC can be conjugated to form malonyl-ACC (MACC) by the enzyme ACC malonyl transferase and recently a new conjugate, glutamyl-ACC (GACC), has been identified in tomato (Martin *et al*, 1995). It has been shown in tomato and grapefruit that as ethylene production increases during ripening MACC levels progressively increase. No biological role is known for MACC or GACC. It remains to be determined what regulates accumulation of these conjugates and if it is a mechanism for limiting the amount of substrate available to ACC oxidase. Conjugation of ACC may be one way of sequestering the precursor to prevent its accumulation and conversion to ethylene.

- **Auto-inhibition:** Under some circumstances biosynthesis of ethylene can be inhibited by applied ethylene in fruit tissue. For example, ethylene treatment has been shown to inhibit wound ethylene production in banana and citrus. Also in fig, treatment with ethylene caused an immediate and complete cessation of the fruit's own ethylene production but upon removal of the ethylene by aeration, ethylene production in the fruit tissue is restored. The effect appears to be reversible. By some means there is a limitation in the availability of ACC perhaps by a feedback repression of the synthesis of ACC synthase.

In addition, ethylene production in ripening fruit may be regulated by interaction with other compounds. It has been shown in several fruit that ripening and ethylene production is promoted when fruit are removed from the tree. These "tree factors" are naturally produced anti-ripening compounds and in apple they appeared to control only ethylene production (Abeles *et al*, 1992). It is not known if tree factors are hormones -

auxin, cytokinin or another compound, or if detachment results in a stress condition which then induces ethylene production and ripening (Abeles *et al*, 1992). In fruit tissues auxin generally inhibits ethylene production (Mattoo & White, 1991), but, in some, auxin is an inducer of ACC synthase. Studies on the peach and apple where high IAA levels precede the burst of ethylene at the climacteric imply that auxin can induce a ripening-related ACC synthase. It is also possible that there is a link between polyamine levels (putrescine, spermine, spermidine) and the plant's ability to produce ethylene. There may be an endogenous regulation between the two biosynthetic pathways because ethylene and polyamines both share S-adenosyl methionine as a common intermediate. In both avocado and tomato an inverse relationship between polyamines and ethylene production has been reported, and a slow-ripening tomato cultivar with reduced ethylene production showed 6-fold higher levels of polyamines than normal wild-type varieties (Khushad *et al*, 1988; Saftner & Baldi, 1990). It is clear that as a young climacteric fruit ages and matures it becomes increasingly sensitive to ethylene (for example in apple, avocado, banana) which reflects its changing physiological state. It has been suggested by Picton *et al* (1995b) that polyamines are candidates for "Ripening-Factor X" which inhibits ripening and decreases sensitivity to ethylene.

In 1972 McMurchie *et al* proposed a model of two systems of regulation that control ethylene biosynthesis in ripening fruit. System I which is responsible for the low rate of ethylene production during growth including stress-related (for example, wounding) ethylene production, and System II which regulates auto-stimulation (autocatalysis) of ethylene during ripening. Climacteric fruit have both Systems I and II, whilst non-climacteric fruit have only System I. The concept of the two systems for the control of ethylene synthesis has been a useful one and molecular biology is beginning to define the genetic control underlying these "systems". It is now clear that growth, development including ripening, and a variety of external factors (stress, wounding) can all determine differential expression of multigene families. System I, then, may represent activation of different ACC synthase and oxidase genes from those of System II. In apple, zucchini, tomato and winter squash ACC synthase gene expression has been shown to be encoded by a divergent multigene family (Rottman *et al*, 1991; Zarembinski & Theologis, 1994) and ACC oxidase is encoded by another multigene family which is also induced during fruit ripening. The ACC synthase of tomato is encoded by at least nine genes, two of which are induced only during fruit ripening, six others are induced by auxin and some

are induced by wounding (Kawakita & Theologis, unpublished, see Zarembinski & Theologis, 1994); two (*LE-ACS2* and *LE-ACS4*) are expressed at the climacteric and during ripening. Experiments with antisense *LE-ACS2* plants in which the expression of both *LE-ACS2* and *LE-ACS4* genes was completely inhibited showed no climacteric rise in ethylene or respiration. In fact, these fruits produced very little ethylene and did not ripen. That is, System II ethylene production is apparently under the control of these specific genes. In time, no doubt, the terms climacteric and non-climacteric fruit, System I and System II ethylene biosynthesis will be more clearly defined in terms of their genetic regulation.

Ethylene, once synthesized, transcriptionally activates a large set of genes. Recombinant DNA technology has allowed isolation and characterization of a number of the ethylene-regulated genes and studies are in progress to elucidate the mechanism of ethylene action at the level of gene expression. Again, the climacteric fruit, tomato, has been used in these studies. cDNA libraries have been constructed using mRNA extracted from either ripe or early mature green fruit treated with applied ethylene. By using either differential or subtractive hybridization techniques or immunological detection methods a number of ethylene and/or ripening induced cDNA clones have been identified (Broglie & Broglie, 1991). In addition, the *LE-ACS2* antisense plants provided a means to determine, by comparison with wild-type fruits, which gene expressions were ethylene-dependent and which were ethylene-independent (or developmentally controlled = ripening related only). These studies have shown that the products of some genes (for example, E4 and E8) accumulate coincident with the onset of ethylene production and their transcription is controlled by ethylene during tomato fruit ripening. The function of the E8 gene and its protein has been determined using transgenic tomato plants expressing antisense E8. They produce 10-fold higher levels of ethylene in fruit tissue only (also co-suppression of this gene leads to a reduction of the E8 protein and elevated levels of ethylene during ripening). This suggests that the E8 gene product is a negative regulator participating in feedback regulation of ethylene biosynthesis in fruit tissue during ripening (Peñarrubia *et al*, 1992). It may be an important component of a signal transduction pathway by which autoinhibition of ethylene occurs (see above) (Fluhr and Mattoo, 1996). The genes that are induced by ethylene are also under complex control mechanisms and efforts are being made to identify which *cis* acting promoter elements in the genes confer ethylene inducibility. In the case of the E4 gene, it has been found that for ethylene-

responsive transcription at least 2 *cis* elements in the gene are required, one of which interacts with an E4/E8 DNA-binding protein which in turn influences the binding of RNA polymerase to the gene (Xu *et al*, 1996).

Ethylene has a continuing role in advancing the ripening process, not just in its initiation. This was observed with experiments using silver ion, an inhibitor of ethylene action. In mature-green tissue of tomato and banana treated with silver thiosulphate ripening is completely inhibited and applied ethylene does not overcome this inhibition (McGlasson, 1985). In fact, in the tomato (a climacteric fruit) treatment with silver halted the ripening process even after it was well advanced (Tucker & Brady, 1987). Also, when the ripening-inhibited *LE-ACS2* antisense tomato plants were treated with ethylene for six days or more, the fruit ripened. These experiments indicate that ethylene must be present continuously to maintain a rapidly turning over set of mRNAs and proteins which initiate the ripening process (Zarembinski & Theologis, 1994). Ethylene not only has a continuing role in regulating transcription at ripening but also it can act at the post-transcriptional level by regulating mRNA translation. For example, antisense *LE-ACS2* and antisense ACC oxidase tomato fruit accumulated PG mRNA but not the PG protein itself. Application of ethylene to these antisense plants led to the renewed accumulation of the PG protein (Oeller *et al*, 1991; Theologis, 1992).

In addition to the synthesis of ethylene, perception of the hormone by ripening tissues is required. Ethylene is hypothesized to bind to its protein receptor most likely through a transition metal (such as copper or zinc) and a cysteine, histidine or methionine residue. When a specific Cys was converted to Tyr or Ser in the ethylene receptor (ETR1) expressed in transgenic yeast, the mutant proteins showed no detectable ethylene binding (Schaller and Bleecker, 1995). An advance in the elucidation of the means by which plants recognise and transduce the ethylene signal was reported by Wilkinson *et al* (1995). They have identified a protein in tomato homologous to the ethylene receptor ETR1 in *Arabidopsis thaliana*. They used the *Nr* tomato mutant which, although it produces ethylene levels similar to normal fruit at the climacteric, is insensitive to the hormone and manifests significant delays in fruit ripening, flower senescence and floral abscission. It was found that the molecular basis of this ethylene insensitivity in the tomato was caused by a single amino acid change in the protein homologous to the *Arabidopsis* ethylene receptor. In addition, this tomato ethylene receptor (NR) was ethylene-inducible in mature-green fruit but not in immature fruit and it was suggested that the regulated

expression of *NR* modulates the observed differential ethylene sensitivity of maturing fruits.

In recent years multiple plant genes have been identified that are homologous to genes involved in animal signal transduction but their function in plants is largely unknown (Braun & Walker, 1996). However, the ethylene signal transduction pathway in plants such as *Arabidopsis* and tomato is gradually unfolding and revealing a novel combination of signalling components with parallels in bacterial, fungal and animal signalling mechanisms. ETR1 appears to be similar to bacterial two-component regulators which act in pairs to control responses to specific signals. Downstream a putative protein kinase, CTR1, similar to an animal Raf protein kinase, regulates a further phosphorylation-dephosphorylation cascade with possibly Ca^{2+} as a second messenger (Chang, 1996).

Thus, a major role for the plant hormone ethylene in ripening of climacteric fruit has been confirmed at the molecular level. In most climacteric fruit sensitivity to ethylene increases with time after anthesis. Cantaloupe and banana will ripen if exposed to sufficient ethylene at any stage, less sensitive fruit such as tomato and apple will not ripen immediately in response to ethylene unless they are close to full maturity and the avocado does not ripen while still attached to the tree but sensitivity to ethylene increases with time after harvest (Brady, 1987; Brady & Speirs, 1991). Also, the variation in amount of ethylene produced by ripening fruit can be quite large even within a species. For example, in apple, some cultivars (Cox's Orange Pippin) exhibit a 40-fold rise in ethylene production during ripening whereas in other cultivars (Golden Delicious, Granny Smith) the ethylene climacteric is small or absent (Abeles *et al*, 1992). In contrast, ethylene seems not to play a large part in the ripening of non-climacteric fruit. These variations in ethylene production and sensitivities may be due to metabolism of ripening inhibitors (auxin, cytokinins, gibberellins, polyamines, "tree factors" etc), or changing concentrations of other hormones (abscisic acid). Species differences in ACS and ACO multigene families and their expression, or even the fruit's ability to acquire and further synthesise the ethylene receptor protein or other regulatory proteins, are all seen as mechanisms for ripening control.

It is clear that ethylene advances ripening in many fruit, that it is necessary for the progression of ripening and the enzymes of ethylene synthesis are induced. Therefore, are there specific elicitors of ethylene synthesis and "signal molecule(s)" which regulate the induction of the chain of ripening events?

Putative Signalling Mechanisms in Fruit Ripening

The initiation of the production of hormones such as ethylene, at the commencement of ripening, would appear to necessitate developmental signals that precede the hormone induction. The elucidation of the signalling mechanisms regulating gene expression in plants is currently the focus of much research (Braun & Walker, 1996).

Signalling molecules suggested include oligosaccharides, polypeptides, fatty acids, galactose, jasmonic acid, calcium and inositol phosphates (Kim *et al*, 1987; Ryan & Farmer, 1991; Schweizer *et al*, 1996). Much of the literature relates to plant defence reactions but plant development, in particular fruit ripening, has been shown to be influenced by some of these molecules. For example, methyl jasmonate promotes ripening in apple fruit and discs (Fan *et al*, 1995) and there are several reports relating to the enhancement of ripening by oligosaccharides and galactose. Increased cell wall hydrolase activity is a prominent feature of ripening fruit and biologically active cell wall-derived oligomers and monomers are thought to be produced in this way. Both climacteric and non-climacteric fruit respond to these cell wall fragments in terms of enhanced ethylene production which suggests that carbohydrate oligomers are utilised as metabolic signals in fruits (Brady & Speirs, 1991). Darvill *et al* (1978) discovered an unusual rhamnogalacturonan (RGII) in the primary cell wall of sycamore, tomato, pea and pinto bean. It had the richest diversity of sugars and linkages known and, since it was too scarce to be a structural polymer, it has been suggested to function as a signal molecule (Carpita and Gibeaut, 1993).

The biological activity of some of these oligosaccharides when applied to mature green fruit has promoted ethylene synthesis and enhanced ripening. For example, pectin oligomers from either ripe tomato or citrus increased ethylene production and stimulated red pigmentation in preclimacteric tomato fruit and discs (Brecht & Huber, 1988; Campbell & Labavitch, 1991). Pectin oligomers extracted from breaker stage fruit (just as ripening begins) also elicited an ethylene increase in mature green discs, and treatment of a Na_2CO_3-soluble pectin fraction from the breaker fruit with pure tomato polygalacturonase generated oligomers active as elicitors of ethylene (Melotto *et al*, 1994). In addition to pectin oligomers, the monomer galactose stimulates ethylene production and promotes ripening when infiltrated into mature green tomato (Gross, 1985) and it has been shown to increase ACC content and ACC synthase activity (Kim *et al*, 1987). This may

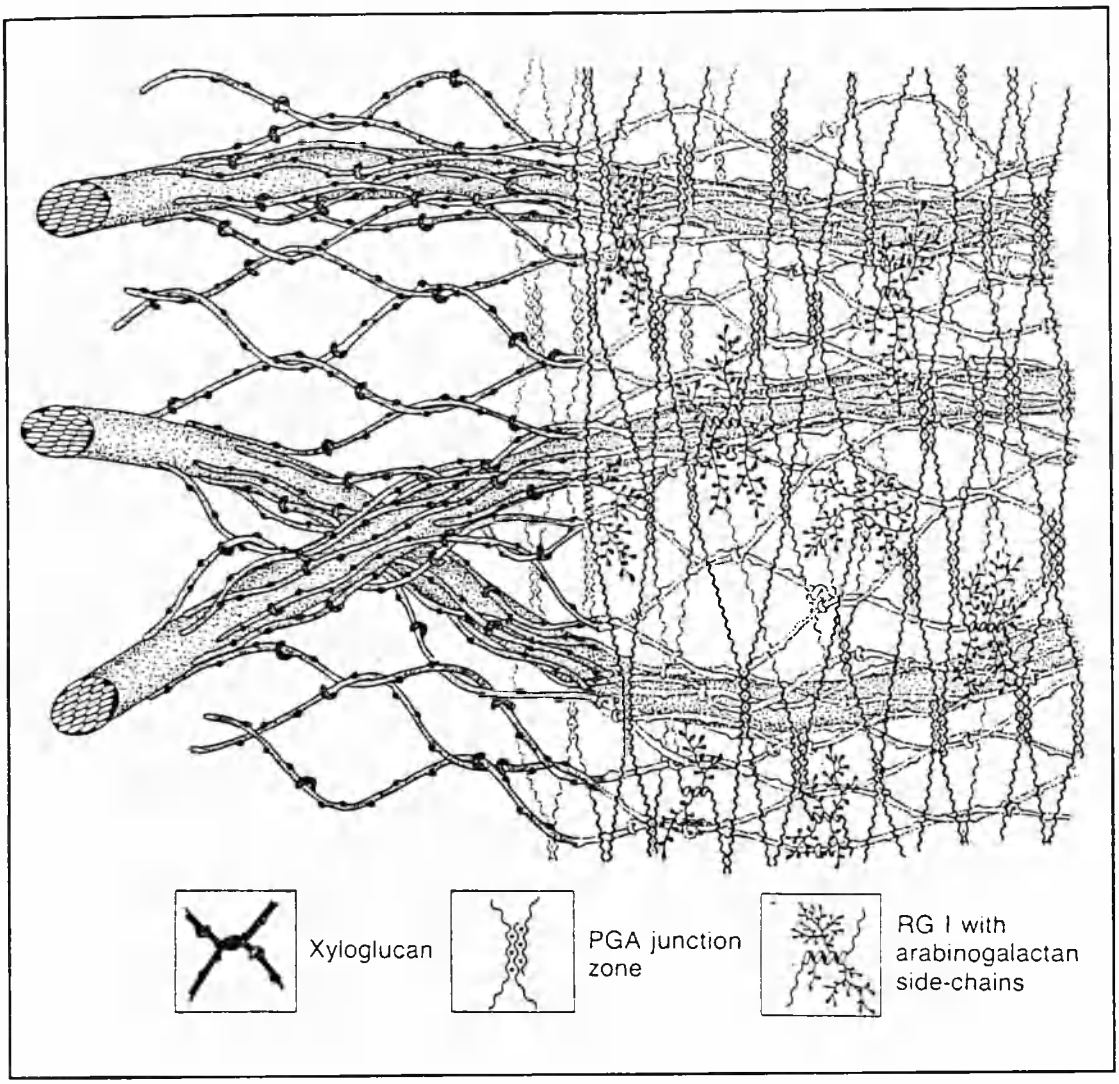


FIG. 1.2: A MODEL OF THE PRIMARY CELL WALL REPRESENTATIVE OF GYMNOSPERMS, DICOTS AND MOST NON-GRAMINACEOUS MONOCOTS
From Carpita & Gibeaut (1993)

Representation of a single stratum of the primary wall just after formation in dividing cells of a meristem. Several such as this coalesce to form a wall. The cellulose microfibrils are interfaced with xyloglucan polymers, and this framework is embedded in a matrix of pectic polysaccharides, polygalacturonic acid and rhamnogalacturonan, the latter substituted with small polymeric side groups of arabinan, galactan, and arabinogalactan. Because xyloglucans have only a single face which can hydrogen bond to another glucan chain, we depict the xyloglucans as woven to interlace the microfibrils. Many other associations are possible, including bridging of two microfibrils by a single xyloglucan. The pectins define the pore size of the wall and this porosity is defined by several factors not fully characterized to date. One of the several possibilities may be the degree of esterification to space the 'junction zones' formed by Ca^{2+}. Another possibility may be that the Ca^{2+} cross-bridging condenses some PGA at the zones but creates larger pores where the intervening chains are separated. Much of the young wall is composed of highly esterified PGA, and the junction zones are enriched in the middle lamellae. The side groups on the rhamnogalacturonan are not well defined and could fulfill biological as well as structural roles.

be physiologically significant since there is a substantial loss of cell wall galactosyl residues during ripening in tomato and other fruits (Gross & Sams, 1984). A class of oligosaccharins which have a ripening-related biological activity in tomato when applied in conjunction with galactose are the N-glycans (Priem & Gross, 1992). They can occur in the free state either by N-glycoprotein proteolysis or as precursors of glycosylation.

These reports demonstrate that a hormonal response can be elicited by carbohydrate oligomers especially in the case of ethylene production. However, the exact details of how these signalling mechanisms might inititate ripening *in vivo* are unclear at the present time.

1.1.2 The Cell Wall of Ripening Fruit and its Enzymes

In ripening fruit the cell wall consists, essentially, of two layers. The more external is the middle lamella which is deposited first at cell division and it connects adjacent cells acting as a kind of cement between them and is composed only of pectic polysaccharides. The next layer is the primary wall deposited whilst the cell is still growing and its general composition in fruits and vegetables is considered to be approximately 35% cellulose, 15% hemicellulose, 40% pectin, 5% protein and 5% phenolics (Brett & Waldron, 1996).

Cell wall structure in ripening fruit has been reviewed by Fischer & Bennett (1991) and Carpita & Gibeaut (1993) have focused on the primary cell wall in their review. They show a 3-dimensional model of the probable interactions between the complex cell wall polymers (Fig. 1.2). Carpita *et al* (1996) summarised recent advances made in the elucidation of this extracellular matrix. They noted that the slow and painstaking chemical analyses of the plant cell wall in the past 40 years have laid a foundation which is now resulting in an understanding of the mechanisms controlling growth and development. It is clear that the cell wall is not the inert structure it was once thought to be but, instead, metabolically highly active and a virtual extension of the cytoplasm. A brief resume of the complex structural polymers of the middle lamella and primary cell wall follows.

- **The Middle Lamella Pectin**

  The types of polymers are the same as those for the primary wall pectin matrix (see below). However, methods to probe the location of the types of pectin have

shown differences between the middle lamella and the primary wall. In carrot and clover root apex gold-labelled antibodies to either unesterified or esterified PGAs revealed that unesterified PGAs were concentrated in the middle lamella (Knox *et al*, 1990; Lynch & Staehelin, 1992). Vreeland *et al* (1989) made a fluorescein-labelled oligogalacturonate probe that bound unesterified PGA in a Ca^{2+} dependent manner and the unesterified pectins were localised in the middle lamella. Selvendran (unpublished observations) noted that cells from mature but unripe fruit (tomato, pear) can be separated with the chelating agent cyclohexanediamine tetraacetic acid at 20°C. This suggests that the ionic bonds between the unesterified regions of pectin in the middle lamella are largely responsible for holding the cells together (MacDougall *et al*, 1995).

- **The Primary Wall Pectin Matrix**

The pectin matrix is composed of two major types of molecule. The first is a helical α-(1,4)-polygalacturonic acid (PGA) homopolymer. Within this PGA homopolymer are α-(1,2)-rhamnosyl residues which serve as attachment sites for side chains of neutral sugars (galactose, arabinose). These residues, often at regular intervals, may delineate PGA homopolymer domains for methyl esterification or de-esterification. These PGA's can condense by crosslinking with Ca^{2+} to form junction zones and link two anti-parallel chains; highly esterified PGA can form parallel chains or there can be an intermediate of partially de-esterified PGA. The second type of pectin molecule is the rod-like heteropolymer rhamnogalacturonan I (RGI) which has a backbone rich in galacturonic acid and rhamnose, and has numerous side chains, attached to the rhamnosyl residues, of arabinose, galactose, arabinogalactan and these side chains themselves may be oligomeric containing xylose, fucose, etc. These complex pectic polymers are thought to perform many functions including determination of wall porosity, providing charged surfaces that modulate pH and ion balance, and serving as recognition molecules that signal appropriate developmental responses. The porosity of the pectin matrix could control movement and activity of enzymes for wall metabolism, and interaction of pectic substances with xyloglucan (XG) may control the access of hydrolytic enzymes to XG substrates.

- **The Primary Wall Cellulose and Hemicellulose**

The cellulose is a linear ß-(1,4)-glucan and the microfibrils it forms become

strongly associated with the hemicellulose. The principal hemicellulose is xyloglucan, a linear ß-(1,4)-glucosyl chain to which xylose and more complex side chains of xylose, galactose and fucose are attached. Other hemicellulosic components include xylans, glucomannans, galactomannans and others. The cellulose-xyloglucan framework is embedded in the pectin matrix and there are complex intermolecular links between these polymers.

- **The Primary Wall Protein**

  The major proteins of the fruit cell wall are the ripening-induced cell wall hydrolases. These have been the subject of intense study since the economically important process of fruit softening was thought to be associated with increased hydrolase activity at ripening. Also, structural proteins, like extensin, have been identified in fruit, e.g. young tomato (Salts *et al*, 1991). The cell wall polymer synthases - cellulose and callose - appear to be plasma membrane-bound rather than located in the cell wall itself (Kudlicka & Brown, 1997).

Fischer & Bennett (1991) note that ripening-associated modifications of hemicellulose structure have been reported in tomato, pepper, strawberry, kiwifruit and melon. In tomato, there was a marked decrease in the size of the hemicellulose polymers as ripening progressed. This suggests that substantial modification of cell wall integrity may occur without manifestation as net changes in cell wall composition (Gross, 1984). Although changes in hemicellulose structure are likely to be an important determinant of textural changes in fruits, it is the ripening-associated changes of the pectins that have been most extensively researched. Ultrastructural studies have shown that in avocado, apple, pear, peach and tomato, as ripening progresses, there is dissolution of the middle lamella and, in some fruit, there appears to be degradation of the fibrillar network of the primary wall. In tomato and avocado, the electron density of the middle lamella decreases and the primary wall tends to swell and appear more fibrous, possibly reflecting a decrease in amorphous materials (Ben-Arie *et al*, 1979, 1989; Platt-Aloia *et al*, 1980; Crookes & Grierson, 1983). These observations at the ultrastructural level seem to be supported by the fact that the average molecular size of pectins decreases greatly during fruit ripening. The extraction of soluble polyuronide, galacturonic acid, and neutral sugars (galactose, arabinose) suggests degradation of pectic crosslinks and of the galacturonan backbone. Cell wall hydrolases, such as polygalacturonase and cellulase, have been shown to accumulate to very high levels in ripening fruit (e.g. tomato and avocado) along with other

exo- and endo-acting glycohydrolases of lesser abundance like α/ß-galactosidase, ß-galactanase, ß-1,3-glucanhydrolase, α/ß-glucosidase, ß-mannanase and α/ß-mannosidase.

- **Cellulase or endo-ß-(1,4)-glucanhydrolase**

  Cellulase activity increases in many fruit during ripening (including avocado, tomato, strawberry, pepper, pear and peach) and in avocado its induction results in prodigious activity. In tomato (Lashbrook & Bennett, 1992), pepper (Ferrarese *et al*, 1995; Harpster *et al*, 1997), and avocado (Tonutti *et al*, 1995), cellulase appears to be encoded by a multigene family that may be differentially expressed during fruit development. Lashbrook *et al* (1994) showed that in the tomato the levels of two endo-ß-(1,4)-glucanhydrolase mRNAs *tomCel1* and *tomCel2* increased during tomato fruit ripening. The mRNA of *tomCel2* did not appear until the initiation of fruit ripening (breaker stage) and reached a greater abundance than *tomCel1* (~20-fold in pericarp). Recently, Gonzalez-Bosch *et al* (1996) have analysed the role of *tomCel1* and *tomCel2* during ripening by comparing their expression in the normal wild type with that in two mutant strains, *rin* and *Nr*. Although it has been reported that the non-softening *rin* mutant possesses near wild type levels of extractable cellulase activity (Brummell *et al*, 1994 and references therein), the estimation of total activity does not reveal the absence of a specific gene product. However, the study of the differential expression of cellulase genes during ripening (Gonzalez-Bosch *et al*, 1996) shows that, in fruit with the *rin* mutation, the expression of the *tomCel2* gene is inhibited, and insensitive to ethylene. In ethylene-treated *rin* fruit the *tomCel1* gene is expressed but it is not sufficient to promote ripening-associated cell wall disassembly or fruit softening, suggesting that the product of the *tomCel2* gene is necessary for this. WE ALSO HAVE FOUND A DIFFERENCE IN THE ABSCISSION ZONE CELLULASE OF THE NON-ABSCINDING KLUANG MUTANT WHEN COMPARED WITH THE NORMAL SEPARATED ZONE and these results are presented in Chapter 5 of this thesis.

  The substrate specificity of cellulase *in vitro* has been examined in avocado. Whilst it does not degrade crystalline cellulose it has activity with substrates such as carboxylmethyl cellulose, mixed linkage ß-(1,3)(1,4)-glucans and has a very limited activity against xyloglucan, a potential *in vivo* substrate (Hatfield & Nevins, 1986). However, although it has been demonstrated that avocado cellulase was not

involved in the ripening-related depolymerization of xyloglucan (O'Donoghue & Huber, 1992), it is possible that cellulase may attack xyloglucan in combination with other enzymes, for example, after glycosidases have reduced its degree of substitution. Also, it may work in conjunction with **xyloglucan endotransglycosylase** (XET). This enzyme is involved in the rearrangement of xyloglucans catalysing the endo-splitting of a xyloglucan and then reconnecting the newly generated reducing end to a non-reducing end of another xyloglucan molecule. Partial lysis of maxtrix polysaccharides during ripening by, for example, xyloglucanase or cellulase, might provide suitable oligosaccharide termini for the enzyme XET. Then polysaccharide to oligosaccharide endotrans-glycosylation would cause a reduction in the M_r (relative molecular mass) of the xyloglucan and a permanent loosening of the wall (Redgwell & Fry, 1993). MacLachlan & Brady (1992) reported that a xyloglucanase in tomato is activated by xyloglucan-derived oligosaccharides. Redgwell & Fry (1993) suggested that this might be endotransglycosylation. In their studies of kiwifruit, they report that XET increased during ripening and visible swelling of the primary cell wall occurred with XET treatment. This indicated that the enzyme may have a key role in early fruit ripening, loosening the cell wall in preparation for further modification by other cell wall-associated enzymes.

- **Polygalacturonase and Pectin Methylesterase**

Both exo- and endo-polygalacturonases (PG) have been identified in ripening fruit tissues of tomato, strawberry, pear, cucumber, papaya, and freestone peach with a specific pattern of expression depending on the fruit. For example, endo-PG in ripe tomato accumulates to high levels but exo-PG is hardly detectable (Pressey, 1987) whereas in ripe apple and strawberry, which were thought not to contain any PG at all, have very low levels of PG activity (Wu *et al*, 1993; Nogata *et al*, 1993). Polygalacturonase hydrolyses the polygalacturonic acid of the pectin whereas pectin methylesterase is the enzyme responsible for de-esterification of the methyl esters in pectin. Pectin methylesterase (PME) has also been found in a large number of fruit. In tomato this enzyme is present throughout development with only a small increase in activity during ripening (Hobson, 1963). Both PG and PME appear to be involved in changes in pectin structure and it has been suggested that they work co-operatively during fruit softening (Huber, 1983).

For many years it was thought that PG alone was primarily responsible for ripening-associated pectin degradation and fruit softening. In tomato, PG is synthesized *de novo* at the onset of ripening, its activity increases dramatically and it shows good correlation with fruit softening (Hobson, 1965). Activity is higher in softer varieties, lower in varieties which soften less and extremely low in the *rin* mutant which barely softens. Also, purified tomato PG causes breakdown of the middle lamella of isolated cell walls and mimics the pectin degradation that occurs *in vivo* (Crookes & Grierson, 1983) However, the use of genetically transformed tomatoes has shown that the effects of polygalacturonase and pectin methylesterase on cell wall degradation and fruit softening are more complex than previously thought. Genetically transformed tomatoes containing antisense PG with about 1% of normal PG activity still ripened and softened with no reduction in the level of soluble polyuronides (although polyuronide molecular weight was greater than in normal) (Smith *et al*, 1988; Gray *et al*, 1994). In addition, Giovannoni *et al* (1989) introduced wild-type levels of PG activity under the control of an ethylene inducible promoter (E8) into tomato fruit of the *rin* mutant which normally has very low levels of PG activity and does not soften appreciably. Even though transgenic fruit had restored PG activity of about 60% of normal (after treatment with an ethylene analogue) they did not soften like wild type fruit. These experiments showed that pectin breakdown alone is not sufficient to cause the degree of softening observed in ripe tomato fruit. It may be that after normal fruit have become ripe the role of PG mediated polyuronide depolymerization is to reduce fruit integrity during senescence (over-ripening).

Transgenic tomato plants expressing an antisense pectin methylesterase gene were also produced and analysed (Tieman *et al*, 1992; Hall *et al*, 1993). PME activity was reduced by about 93% in these fruit which resulted in an increase in the size and esterification of pectin fragments and a large increase in soluble solids. Again, ripening and softening appeared to be unaffected but during senescence the reduced PME fruits exhibited the opposite effects on firmness to those observed in the reduced PG fruit. Whereas an increase in fruit integrity and storage life has been observed in fruits with low PG activity, almost complete loss of fruit integrity is observed in fruits with low PME activity. Thus, PG and PME may have a major role in tissue deterioration during the later stages of ripening.

- **ß-Galactosidase/ß-Galactanase**

  These enzymes degrade the ß-galactosyl residues of the neutral sugar side chains attached to rhamnosyl units in the main galacturonan backbone. In tomato, and other fruit, chains of ß-(1,4)-galactan are common (Carpita & Gibeaut, 1993; Carey *et al*, 1995). In muskmelon, persimmon and mango there is little or no detectable PG activity, yet these fruit still soften during ripening. Since PG has been shown not to be a primary determinant in the softening of tomato fruit, attention is now focusing on other hydrolases, in particular, ß-galactosidase/ galactanase. Recently, these enzymes have been studied in many ripening fruit, including tomato (Carey *et al*, 1995; Carrington & Pressey, 1996), kiwifruit (Wegrzyn & MacRae, 1992), persimmon (Cutillas-Iturralde *et al*, 1993), apple (Ross *et al*, 1994), papaya (Lazan *et al*, 1995), muskmelon (Ranwala *et al*, 1992), mango (Ali *et al*, 1995), avocado (de Veau *et al*, 1993) and Japanese pear (Kitagawa *et al*, 1995). The role of these enzymes in ripening is unknown but they are speculated to play a key part in fruit softening (Carey *et al*, 1995). A ß-galactosidase has been reported to degrade and solubilise pectin (de Veau *et al*, 1993) by releasing monomeric galactose. The increase in solubility was suggested to be due to a decrease in the ability of pectin molecules to aggregate together. In addition, galactose increases 4 to 6-fold during tomato fruit ripening (Gross, 1983) and, as discussed in Section 1.1.1, can stimulate ethylene production and the biological activity of N-glycans. Thus, ß-galactosidase action may be relevant to other ripening processes as well as fruit softening.

- **ß-(1,3)-Glucanhydrolase (Laminarinase)**

  The increased activity of this enzyme has been identified in ripening tomato and peaches (Hinton & Pressey, 1980) and is present in many other fruits. Evidence for structural ß-(1,3)-glucans in walls of fruits is lacking (Labavitch, 1981) and, like cellulase, its *in vivo* substrate is unclear. Fry (1995) notes that an exo-*O*-glucosyl hydrolase which catalysed the transfer of single glucose units also catalysed the hydrolysis of laminarin. It may be that, *in vivo*, this enzyme takes part in transglycosylation reactions rather than hydrolytic activity. ß-1,3-glucanase activity increases in response to ethylene, pathogen attack and pathogen-derived elicitors. However, ß-1,3-glucanase has been shown to accumulate in healthy tissue during development (Lotan *et al*, 1989). Vögeli-Lange *et al* (1994) reported

that in the germination of tobacco seeds there was a highly localised expression of ß-1,3-glucanase in the endosperm prior to penetration of the radicle through it. This suggests that ß-1,3-glucanse is involved in lowering the mechanical constraint presented by the endosperm on the embryo and it acted to "soften" or weaken the tissues during radicle protrusion (Black, 1996).

- **Other Cell Wall Hydrolases**

  Many other enzymes have been reported to be present in ripening fruit and the complexity of cell wall polysaccharide suggests that they have a role to play in its ripening changes. These include glycosidases such as α-galactosidase, α/ß-mannosidase, α/ß-glucosidase, α/ß-xylosidase/xylanase (Fisher & Bennett, 1991). An α-arabinofuranosidase was reported to increase 15-fold during ripening of the Japanese pear (Tateishi *et al*, 1996) and endo-ß-(1,4)-mannanase also shows increased activity during ripening of tomato (Pressey, 1989).

The data collected on cell wall changes and enzyme activities during fruit ripening suggest softening of fruits is a more complex process than anticipated and that the actual composition of the cell wall plays a critical part in tissue softening. Comparisons have been made with soft and firm fruits of the same variety. Batisse *et al* (1996) have shown that the major difference between soft and crisp cherry fruit was in the degree of polymerization of pectin side chains. Crisp fruit had a high degree of polymerization with numerous bonds between the polymers and soft fruit possessed fewer interactions between polymers. Mature green crisp mesocarp had thick primary walls with numerous large spaces between cells whereas soft mesocarp showed cells larger than the crisp fruit, distorted with thin undulated primary walls and few intercellular spaces. Another example is the tomato mutant of the cultivar Manapal (known as *dg*) which has deeper red, smaller and firmer fruit than the parent strain. The increase in PG activity was similar in both types of fruit but it was found that *dg* fruit had twice as much cell wall material as the parent strain (Tong & Gross, 1989). An histological study of firm and soft ripe dates showed that firm dates had rigid intact cell walls similar to the immature tissues, whereas soft dates had lost considerable cell wall structure and contained many broken cells (Hasegawa *et al*, 1969 and references therein).

It is possible that in softening fruits there is a dynamic turnover of cell wall components with cell wall polymer association changes rather than a simple process of cell

wall and middle lamella degradation of long-existing wall components. Knee & Bartley (1981) have suggested that wall turnover in fruit softening is initiated by wall synthesis. Pectin solubilisation could be due to *de novo* synthesis of highly methylesterified polyuronide concurrently with degradation of low esterified regions of middle lamella pectin. The new pectin because of its higher degree of methylation (DM) would be less strongly bound in the middle lamella by ionic bonds and this could lead to cell separation. This dissolution is accompanied by the loss of galactan and arabinan side chains and softening could result from changes in the interactions between various wall polymers.

Finally, fruit softening can result in part from the starch hydrolysis during ripening and this has been observed in banana, apple, kiwifruit, mangoes and jackfruit (Tucker & Grierson, 1987; Abeles *et al*, 1992; Rahman *et al*, 1995). In addition, a decrease in turgor is said to contribute to ripening-related softening (Shackel *et al*, 1991; Mignani *et al*, 1995).

Whilst the basis of softening and texture changes in ripening fruit is yet to be fully elucidated, the alterations in the structure of the cell wall and the enzymes involved in these changes, appear to occur, according to Brady (1987), not only in fruit but also probably in the abscission zone. Addicott (1982) notes that the ripening and softening of fruit seems to involve biochemical and cytological changes which are very similar to the changes observed in cell separation of the abscission zone. Therefore, the foregoing information is very relevant to work in this thesis, as well as the following section on Abscission.

1.2 ABSCISSION : ESPECIALLY ABSCISSION OF FRUITS

Abscission (Latin: *ab* = off, away, from; *scindere* = to cut asunder, OED, 1989) is the organised shedding of part of a plant and the term usually refers to this special process in plants only. Mostly, this involves the shedding of fruits, leaves, flowers and petals. In horticulture and agriculture enhancement of abscission can be used to advantage in the thinning of flowers or fruit, to facilitate mechanical harvesting by loosening ripe fruit and in the controlled shedding of leaves prior to normal leaf drop (Lürssen, 1991). In spite of the economic relevance of abscission in fruits this phenomenon has mainly been studied in leaves (for example, bean, elder, citrus), although it appears that the process of fruit abscission is essentially similar to that of leaves and other organs. It is a

metabolically determined programme of cell separation usually between cells at precisely defined locations at the base of the organ to be shed (Osborne, 1989).

1.2.1 The Abscission Zone

The abscission zone of fruits is often between the **fruit and pedicel** (oil palm, mango, squirting cucumber, avocado, melon, citrus and wild-oat) or **pedicel and stem** (apple, cherry, olive, pear). Some fruits have abscission zones at both positions (peach, avocado, orange) whilst others have an abscission zone at a different anatomical site, for example, between the fruit and receptacle (red raspberry) or the dehiscent pod which releases the seed=fruit (rape, okra, pea). The anatomy of the fruit abscission zone has been documented at the light microscope as well as ultrastructural levels for a number of fruit including the peach (Rascio *et al*, 1985), oil palm (Henderson & Osborne, 1990), wild-oat (Sargent *et al*, 1984), olive (Reed & Hartmann, 1976), lemon (Iwahori & van Steveninck, 1976) and rape-pod (Meakin & Roberts, 1990a; 1990b), but many studies have been restricted to the light microscope level, for example, squirting cucumber (Wong & Osborne, 1978), cherry (Stösser, 1969), apple (Pandita & Jindal, 1991), red raspberry (MacKenzie, 1979; Sexton *et al*, 1997), and orange (Wilson & Hendershott, 1968).

In a study of the tomato "knuckle" zone where the fruit stalk and pedicel join, it was suggested that the abscission region may have additional functions (Lee, 1989). At this position, there is a significant reduction of xylem and a major increase in phloem cross-sectional area through the abscission zone. This was said to produce a high resistance to water flow in the xylem and transfer the demand for water by the fruit to the phloem. This would then link water with dry matter influx into the fruit. Transpiration from the fruit would then play a role in maintaining the flow of phloem sap to the fruit. However, there is clearly no difference in the ability to transport auxin at this position, either acropetally or basipetally, in bean abscission zones of petiole explants (Jacobs *et al*, 1966), suggesting that there is no special impediment or facility at the zone to the polar transport mechanism.

Abscission occurs by dissolution of the middle lamellae joining the specialised group of cells which form the abscission zone. The zone usually constitutes a plate of 1-2 cell layers across the base of the organ to be shed but in some species a wide multicellular plate up to 50 cells wide is differentiated. The cells may appear similar to those of

surrounding tissue, but frequently they can be distinguished as a smaller closely-packed layer with dense cytoplasmic contents. In the squirting cucumber the functional cells of the abscission zone at the ovary-pedicel junction of young flower buds were identified by their 8C nuclear DNA content whereas surrounding non-abscinding tissue was 4C (Wong & Osborne, 1978).

Separation of the abscission zone usually begins from a single point of origin and spreads throughout cells of the zone. THIS IS ESPECIALLY EVIDENT IN THE ABSCISSION OF OIL PALM FRUIT (see Section 1.3.2). Addicott (1982) suggests the following sequence of anatomical changes which appears to be similar in abscission zones of fruit, flower and leaf.

- The **middle lamella** appears to swell considerably and, finally, it weakens and separates. When the middle lamella of two or more cell layers is involved, some cells separate completely and appear as isolated intact single cells. Abscission cells also expand exerting a mechanical strain upon the abscission layer. Hydrolysis of accumulated starch (often seen in pre-abscission cells) occurs at the induction of abscission and may well provide the soluble sugars to cause these turgor changes at the zone (Osborne, 1989), or else the starch may be utilised as a source of energy. Freshly separated surfaces of abscission zones reveal intact and somewhat rounded or elongated cells. WE HAVE OBSERVED THESE EVENTS IN OUR SCANNING ELECTRON MICROGRAPHS OF OIL PALM ABSCISSION ZONES (Henderson & Osborne, 1990, and Section 3.2.1).

- Although **primary cell wall** polymer changes may occur during cell expansion, primary wall disintegration is not observed.

- The **vascular traces** are the last to separate but the actual mechanism by which this occurs is unclear. It is possible that it is simply mechanical breakage after the separation of the surrounding parenchyma cells and the pressure exerted by cell expansion. Also, tylose penetration can weaken and distort the walls of xylem vessels making them susceptible to fracture. Enzymatic hydrolysis of the middle lamellae between end walls of xylem elements would also facilitate their separation. Whilst some softening of lignified secondary walls during abscission has been recognised for some time, the presence and activity of lignases has not

Ultrastructural changes within the abscission zone itself just prior to separation include an increase in ribosomes, polysomes, rough endoplasmic reticulum and dilation of the membranes of the dictyosome stacks with subsequent dictyosomal vesicle formation. The vesicles containing the products of these syntheses fuse with the plasma membrane and are discharged into the apoplast. These observations indicate an increased level of protein synthesis and secretion (Osborne, 1989). In a recent study of the peach fruit abscission zone, before and after separation, changes in nuclear morphology were observed (Tirlapur *et al*, 1995). Confocal laser scanning microscopy of pre-abscission zone cells revealed well organised large nuclei but during abscission there was a reduction in nuclear volume, fragmentation of DNA and a decrease in ethidium bromide fluorescence. The cell death in peach abscission cells was suggested to be analogous to the situation during apoptosis or programmed cell death in animal systems (apoptosis in *Euglena gracilis* had been reported by Tirlapur also). Osborne & McManus (1986) showed that abscission cells are at a terminal stage of differentiation but in bean only the distal side of the abscission zone shows nuclear pycnosis in the short term (McManus *et al*, 1998). THIS CONCEPT IS CONSISTENT WITH OUR FAILED ATTEMPTS TO FORM A CALLUS TISSUE CULTURE FROM OIL PALM ABSCISSION ZONE CELLS. DESPITE MANY EFFORTS THESE CELLS ALWAYS BECAME SENESCENT AND DIED, whereas Unilever were able successfully to generate an oil palm callus from the root tissue and from veins of young leaves and young inflorescences (Jones, 1983).

1.2.2 The Biochemistry and Regulation of Abscission

The promotion of abscission by ethylene and its inhibition by auxin is well documented (Osborne, 1989). In the early stages of fruit development auxin levels are usually high but ethylene production can be high too. At this time in fruit development the relative levels of auxin and ethylene can be crucial to the continued attachment of young fruits, although other factors may also be involved. In general, it appears that high auxin and low ethylene concentrations in the fruit are required for the prevention of early fruit drop (McGlasson *et al*, 1978; Brady & Speirs, 1991), and the reverse usually occurs in abscission of mature fruits.

Ripe climacteric fruits which mature on the plant produce ethylene (Section 1.2.1) and this is believed to be one of the most important signals in promoting abscission. The muskmelon (netted variety) is one example of this. Ethylene levels increase during ripening but the peak of ethylene production occurs at fruit maturity and abscission from the vine (Abeles *et al*, 1992; Ranwala *et al,* 1992; Dunlap *et al*, 1996; Aggelis *et al*, 1997). In the oil palm, although ethylene levels are undetectable during ripening, it too has a surge of ethylene production at full fruit ripeness and abscinds within a few hours of the onset of ethylene production. However, unlike the unripe melon (which will ripen and soften with applied ethylene after harvesting), unripe oil palm fruit, once harvested, will separate at the abscission zone but will never ripen (see Chapter 4).

It is of interest to note that ethylene does not enhance abscission in all fruits. The shedding of the ripe fruit of wild-oat for example, is not promoted by ethylene and the ethylene inhibitor AVG did not delay abscission either (Sargent *et al*, 1984). The ultrastructural changes of the middle lamella at separation were shown to be similar to those in ethylene-induced abscission zones but in the wild-oat abscisic acid, and not ethylene, accelerated the separation process.

As the abscission process progresses, fruit "loosen". This loosening culminates in abscission but if the cell separation has not proceeded too far, then it has been shown to be reversible in some cases. Abeles *et al* (1992) gives two examples in fruits. In apples application of an ethylene releasing compound caused a decrease in the force required to remove them, but a few days later the force required to remove them returned to normal. Valencia oranges loosened and abscinded when ripe, but fruit which did not abscind at this point regained the structural integrity of their abscission zones until abscission at fruit senescence. This effect in citrus also has been observed when abscission chemicals have been applied in the field (Holm & Wilson, 1977). The process of "re-tightening" occurs not only in the abscission zones of fruits but in leaves as well, and Osborne (1989) has summarised this concept of two stages in the abscission process. Stage 1 was either non-responsive or poorly responsive to low levels of ethylene and the duration of this phase was determined by the amount of auxin in the zone. Once repressive levels of auxin fell below a critical level and depending upon the concentration of ethylene present, Stage 2 was initiated. This second stage included activation of new genes, and production of zone-specific glycanhydrolases resulting in the loosening of middle lamellae and cell walls. These processes are observed as a "loosening" of fruit, or a decrease in the

"breakstrength", but once Stage 2 has progressed beyond a certain degree of cell dissociation, abscission becomes irreversible.

The abscission zone cells have a unique ability to sense and respond to abscission signal(s) by initiating a gene expression programme that leads to separation. Incorporation of radioactive precursors into RNA and protein was increased by ethylene and confined to the cells undergoing separation that were on the proximal (retained) side of the zone (Osborne, 1968). In addition, RNA and protein synthesis were shown to be necessary for abscission since it was inhibited by cycloheximide and actinomycin D (Abeles *et al*, 1992); application of cycloheximide to cherry and orange fruit inhibited abscission (Wittenbach & Bukovac, 1973; Greenberg *et al*, 1975). Investigation of this *de novo* protein synthesis has focused on the enzymes endo-ß-(1,4)-glucanhydrolase (cellulase) and polygalacturonase. However, the many other hydrolases which have been examined in the flesh of ripening fruit, such as ß-galactosidase and ß-(1,3)-glucanhydrolase, have not been as intensively studied in fruit abscission zones.

- **Cellulase**

  In the bean (petiolar leaf explants) it was shown that ethylene enhances cellulase activity and auxin represses its activity. Non-zone and zone cells were shown to have a constitutive cellulase of pI 4.5 whereas in zone cells a specific isoenzyme of pI 9.5 appears *de novo* in response to ethylene exposure at abscission. The increase in mRNA and protein for this pI 9.5 isoenzyme was found to correlate closely with the initiation and progress of abscission (Osborne, 1989 and references therein). In fact, Sexton *et al* (1980) prevented abscission by treatment of the zone with an antibody they raised to the purified pI 9.5 cellulase indicating a role in cell separation. Detailed studies of the spatial distribution of this enzyme during ethylene-initiated abscission demonstrated that it begins to accumulate in the vascular bundle parenchyma cells of the abscission zone and pulvinus, and progresses outward through the cortical cells of the abscission zone (Sexton *et al*, 1981; del Campillo *et al*, 1990). Subsequently, Thompson & Osborne (1994) showed that the vascular system is responsible for the production of signal molecule(s) essential for both cell separation and expression of the pI 9.5 cellulase in cortical cells of the abscission zone.

A cDNA clone to bean abscission pI 9.5 cellulase was used to confirm that it did not share sequence similarity with the pI 4.5 cellulase. The cDNA probe also showed that ethylene initiated, and was necessary for, the continued accumulation of the mRNA for the pI 9.5 protein. When explants were treated with auxin prior to ethylene exposure, cellulase mRNA accumulation was inhibited as was the expression of cellulase activity (Tucker *et al*, 1988). Osborne *et al* (1985) had shown that auxin negatively regulates cellulase production throughout the abscission process and this too confirmed the opposing stimulatory and inhibitory effects of ethylene and auxin.

The physiology of leaf and fruit abscission is similar and, although fruit abscission has not been as extensively studied as it has been in the leaf, cellulases have been identified in fruit abscission zones. They include peach (Rascio *et al*, 1985), rape pod (Meakin & Roberts, 1990b), orange (Goren *et al*, 1973; Greenberg *et al*, 1975), red raspberry, (Sexton *et al*, 1997), avocado (Tonutti *et al*, 1995), tomato flower (Tucker *et al*, 1984; Gonzalez-Bosch *et al*, 1997), apple (Kondo & Takahashi, 1989), and squirting cucumber (Jackson *et al*, 1972). The question arises - are the same genes involved in the response to the regulatory signals in leaf-fall, fruit abscission and fruit ripening?

Recently, molecular biological techniques have been used to compare the expression of cellulase genes in fruit tissue with those in their abscission zones. In ripe avocado mesocarp, there are 2 distinct genes *Cel*1 and *Cel*2 which hybridise to a ripening-induced cellulase cDNA, and the *Cel*1 gene sequence matches a number of cellulase cDNAs isolated from ripe avocado fruit libraries. The activated abscission zone also accumulates *Cel*1 mRNA which suggests that the same gene is involved in fruit ripening and abscission (Tonutti *et al*, 1995). Bonghi *et al* (1992) have used an avocado fruit cellulase cDNA clone in a northern analysis for the cellulase expressed in peach pedicel-fruit abscission zones. Two hybridising mRNAs were detected and ethylene treatment increased the level of one of them.

The cellulases of the tomato flower abscission zone (the presumptive "knuckle" of the fruit abscission zone) have been examined in comparison with those expressed in ripening fruit. Lashbrook *et al* (1994) found that the expression of *Cel*1 and

*Cel*2 overlap in flower abscission zones and ripening fruit - both mRNAs are present but their relative abundance is different; *Cel*1 mRNA is the more abundant in flower abscission zones, whereas *Cel*2 mRNA predominates in ripening fruit. *Cel*1 shows a high degree of amino acid sequence identity to the bean pI 9.5 cellulase, suggesting that these two cellulases share similar functions. Specific antibodies raised against *Cel*1 confirm that the accumulation of *Cel*1 protein correlates with the progressive increase in the proportion of abscinding flowers (Gonzalez-Bosch *et al*, 1997). However, *Cel*1 expression in antisense plants resulted in only a partial reduction in flower abscission. The authors suggested that *Cel*1 does not act alone and progression of abscission may require accumulation of several cell wall hydrolases, including *Cel*1 at the final stages. Further research by the same group (del Campillo & Bennett, 1996) showed that in ethylene-treated zones, 6 cellulase genes have been isolated from the total RNA and 4 were homologous to fruit pericarp cellulases. The expression of the two other genes specific to the zone, *Cel*5 and *Cel*6, correlated with natural and ethylene-induced flower shedding. *Cel*5 mRNA increased in the later stages of abscission and *Cel*6 mRNA was present in non-abscinding zones decreasing in the final stage of abscission.

Studies such as these will no doubt offer opportunities to distinguish between the genes involved in cell-wall weakening and those involved with cell wall reorganisation. The exact site of cellulase action on the abscission zone cell wall is not known and cellulase is unlikely to be the sole enzyme involved in the abscission process.

- **Polygalacturonase**

Despite the fact that ultrastructural studies reveal degradation of middle lamella pectin, the polygalacturonases have not been as extensively studied as the cellulases. At times no correlation between PG activity and abscission was observed and sometimes increased PG activity was not detected (Rasmussen, 1973; Berger & Reid, 1979; Meakin & Roberts, 1990b). However, demonstration of increased PG activity at abscission in fruit zones has been reported for peach (Rascio *et al*, 1985), orange (Rogers & Hurley, 1971; Greenberg *et al*, 1975; Basiouny & Biggs, 1976), apple (Pandita & Jindal, 1991), tomato flower (Tucker *et al*, 1984), and an increase of PG transcripts at dehiscence in rape pod (Petersen

et al, 1996). In another cell separation system, the complete release of cells from the root cap in pea (within 30h) was correlated with an induced PG activity (Hawes & Lin, 1990).

There has been a great deal of research on the ripening-related tomato fruit endo-PG (Section 1.2.2) and the ripening PG cDNA probe has been used to determine if the abscission PG is a different gene product. Taylor *et al*, (1990) showed that in tomato leaf abscission zones the cDNA of the fruit PG did not hybridise to mRNA isolated from the abscission zone of the leaves and a polyclonal antibody raised against the fruit PG did not recognise the leaf abscission protein. In addition, leaf abscission was unaffected in the transgenic antisense fruit PG tomato plants. There was increased PG activity as tomato flower abscission zones separated (Tucker *et al*, 1984) and, recently, Kalaitzis *et al* (1995) have characterized a PG cDNA expressed in these zones. They reported that it shares only 43% identity at amino acid level with tomato fruit PG. High levels of expression coincided with the occurrence of abscission after 24 hours of ethylene treatment and correlated with accumulation of the PG mRNA. Leaf petiolar explants were used to examine the expression of TAPG1 when treated with IAA or silver ions. The TAPG1 mRNA was not expressed in either case, and this is consistent with the known effects of silver ion on the perception of ethylene and of IAA on the ethylene induction of the cell wall hydrolases in abscission.

Analysis of the peach fruit-pedicel abscission zone PG by Bonghi *et al* (1992) revealed two exo-PGs and one endo-enzyme. Ethylene treatment activated the endo-enzyme and the high pI exo-enzyme but, surprisingly, depressed activity of the low pI exo-enzyme. A tomato fruit PG cDNA probe hybridised with mRNA of the endo-PG enzyme. The rape-pod mature dehiscence zone has been examined by Petersen *et al* (1996) and a full length cDNA clone of one endo-PG isoform was expressed exclusively in the dehiscence zone late in development.

1.2.3 Mutants of Abscission

Mutants of leaf abscission have been reported for the birch tree (Rinne *et al*, 1992), lupin (Atkins & Pigeaire, 1993; Osborne & Thompson, 1991) and the 'Ricalate' variety of the navel orange (Zacarias *et al*, 1993). Mutants of fruit abscission include the coconut variety 'Malayan Tall' (Dr R.H.V. Corley, personal communication, 1997), the *jointless*

FIG. 1.3: **CHANGES IN THE APPEARANCE OF OIL PALM FRUIT SPIKELETS DURING RIPENING (CLONE 271D)** [A-D approximately half the original size; E about two-thirds original size.]

A. Pre-anthesis (0 daa).

B. Unripe (63 daa); the deep pink-purple colour of the fruit is due to pigment in the exocarp (and not carotene in the mesocarp).

C. Unripe (118 daa); increased carotene synthesis begins around 120 daa.

D & E. Near ripe (140 daa) and fully ripe (150 daa); these fruit show the deep orange colour due to the accumulation of carotene during ripening.

pedicel tomato (Butler, 1936) and the 'Ricalate' variety of navel orange (Zacarias *et al*, 1993). Also, it has been suggested that in the pepper domestication has led to the selection of non-abscinding fruit, whilst other pepper cultivars have deciduous fruit (Abeles *et al*, 1992).

In the *jointless* tomato there is no abscission joint where it normally occurs on the pedicel midway between the peduncle and calyx (although abscission at the base of the leaves progresses normally). However, in the birch, lupin and navel orange mutants, the abnormality is not caused by lack of abscission zone development. In the leaf-petiole abscission zone of the lupin, cellulase activity in the *Abs*$^-$ mutant was lower than in the wild type (Osborne & Thompson, 1991), and both cellulase and polygalacturonase activity were lower in the delayed flower abscission of the mutant tomato Neverripe compared with wild type (Tucker *et al*, 1984).

Non-abscinding palms have been discovered on Unilever plantations in Malaysia and Indonesia. These fruit appear to develop and ripen like the normal clones but at full ripeness the fruit are not shed from the palm (Section 3.2). Another abnormal oil palm fruit is fully "mantled". This fruit has a ring of up to six appendages, known as supplementary carpels, which develop from the stamen primordia or rudimentary androecium in the female flower (Corley *et al*, 1986). Fully mantled fruit do not abscind like normal fruit: 1+2 or 1+3 (Section 1.3.2). These positions have developed into mesocarp tissue, and their separation can only occur 1+4/5, usually at the over-ripe stage (Osborne *et al*, 1992).

1.3 WHAT IS KNOWN ABOUT FRUIT RIPENING AND ABSCISSION IN THE OIL PALM

The oil palm is monoecious, that is, it bears both male and female flowers and pollen from the male flowers pollinates the female flowers, either on the same or more often a different palm, to produce fertile seed. The female inflorescence has a length of 30cm or more before opening up and it consists of numerous spikelets. The spikelets are arranged spirally around a central rachis (a stout peduncle). When mature, these inflorescences are quite large (up to 25 kilograms), each containing about 500-1500 or more fruit (Fig. 1.4). Fruit is produced all the year round but in different amounts at different times. Figure 1.3B shows fruit with purple to black pigmentation (probably

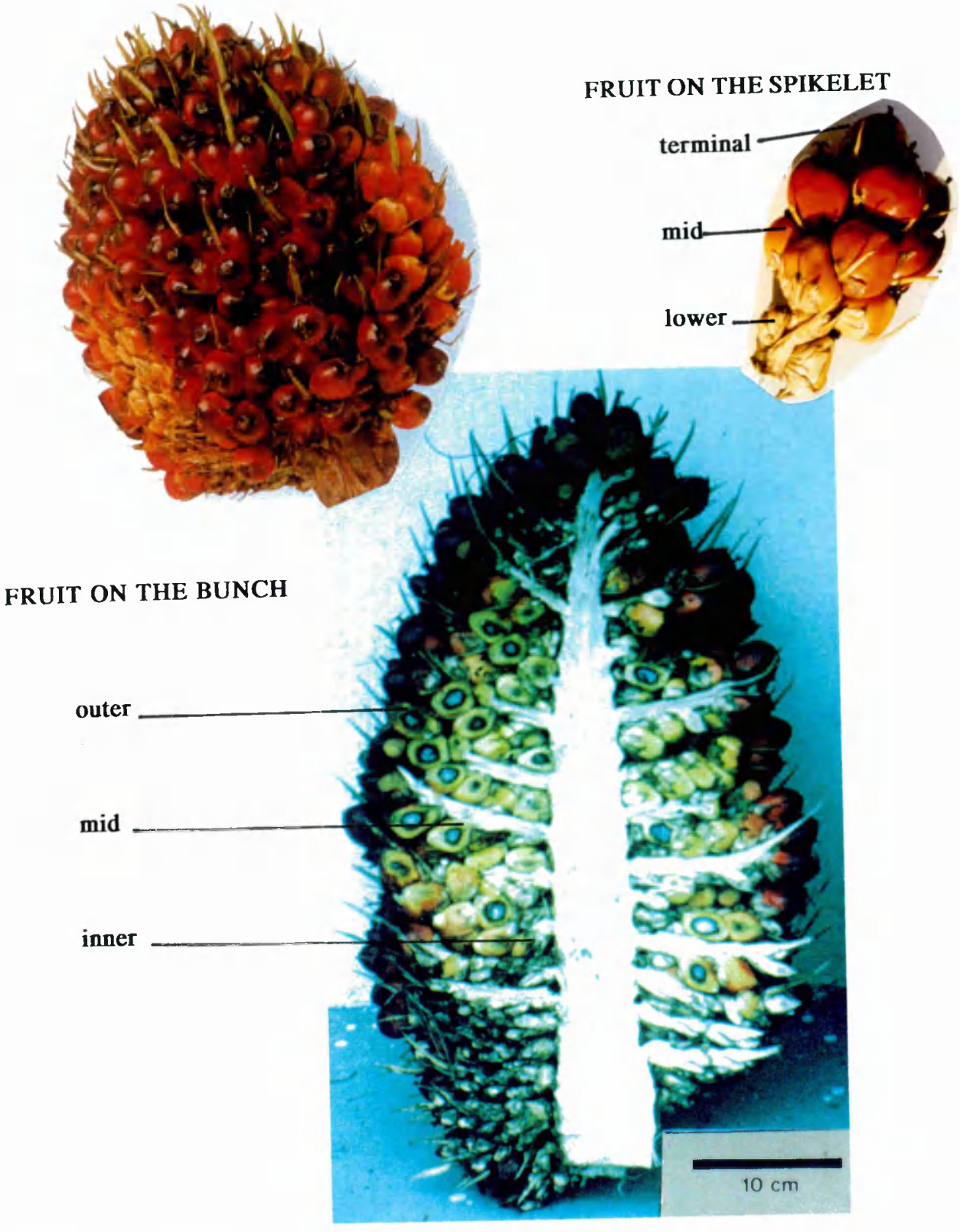


FIG. 1.4: THE OIL PALM FRUIT BUNCH

The "branches" from the stout peduncle (shown in the longitudinal section) are the individual spikelets arranged spirally around the central rachis. Inner fruit are buried deep within the fruit bunch and correspond to the lower fruit on an individual spikelet. Similarly, the outer fruit on the bunch correspond to the terminal fruit on a spikelet.

anthocyanins in the exocarp) characteristic of unripe fruit and this gradually decreases during fruit maturation (Fig. 1.3C). At 120-130 days from anthesis (daa) the fruits are full-sized, pale yellow, and start to ripen (Fig. 1.3C & D). Ripening (observed by the colour change in fruit from pale yellow to orange) is usually from the apex of the bunch downwards and at approximately 150-160 daa, the fruit is generally fully-ripe and fruits from any one inflorescence fall from the bunch over a period of about three weeks. Thus, fruit is ready for harvesting within a period of six months from pollination (Hartley, 1988).

Understandably, the majority of studies on the oil palm have concentrated on its economically valuable oil product so as to obtain the best yields. In the earliest of studies by Frémy and Stenhouse in the 1840s, the first aim was to determine the composition of mesocarp oil. Since then the quest to obtain a high yield of final product and also of high quality (low in FFA and free of its inherent colour) has addressed questions relating to the anatomy, physiology and biochemistry of the oil palm fruit.

1.3.1 Oil Content Changes in Ripening Fruit Mesocarp

The mesocarp oil, which is about 97% triacylglycerol at maturity, is not deposited throughout fruit development but it is synthesized during the final 4-5 weeks of ripening (Fig. 1.5C). There is variation in the quantity of oil produced by individual fruits and this is related to its position in the fruit bunch (Fig. 1.4). Inner and basal fruit have a lower oil content than outer and apical fruit. Inner fruit, or those embedded deep in the bunch cluster, have been found to have a negligible quantity of oil even in ripe fruit (Thomas *et al*, 1971). Jeje *et al* (1978) note that fruit position in the bunch could account, in part, for differing oil content values reported for ripe mesocarp of between 20% to 45%. Oo *et al* (1986) who used outer fruit only in their lipid analyses report that total lipid was 47% of fresh mesocarp weight.

Early anatomical investigations of ripening fruits in 1922 and 1925 revealed that the oil droplets appeared first in tissue near the base of the fruit (just above the abscission zone). This lipid deposition gradually extended radially and apically (Jeje *et al*, 1978). Unfortunately, there are no published ultrastructural studies of oil palm mesocarp comparable to those of the avocado, another oil-bearing fruit. In avocado, oil deposition occurs in mesocarp parenchyma cells and in oil cells. In the parenchyma cells many

individual oil bodies are observed in the cytoplasm, elaioplasts show a coronae of oil droplets and the cell vacuole is small (Murray Scott *et al*, 1963; Platt-Aloia & Thomson, 1981) whereas, the oil cell has a suberised cell wall and is filled with a single large "oil sac" (Platt-Aloia *et al*, 1983).

At the present time, little is known about the pathway of oil palm mesocarp triacylglycerol biosynthesis *in vivo* or its regulation, but Bafor & Osagie (1989) have proposed possible pathways of biosynthesis based on the pattern of the accumulation of the various lipid classes - phospholipids, glycolipids and neutral lipids - during fruit development and maturation. In the early stages of fruit development total lipid is only about 1% of the mesocarp fresh weight of which phospholipids and glycolipids (polar lipids) constitute the major part (>50%) and these are probably required for the cellular proliferation in the growth stage to 120 daa. In mesocarp of mature ripe fruit these polar lipids diminish to less than 2% of the total lipid and neutral (non-polar) lipids (mainly triacylglycerol) increase to 97% of the total mesocarp lipid (Bafor & Osagie, 1986). The overall fatty acid composition of the triacylglycerol in fully ripe fruit is shown in Table 1.1.

FATTY ACID		% FATTY ACID TYPE IN MESOCARP FROM:		
		MALAYSIA	AFRICA	INDIA
		Oo *et al*, 1986	Bafor & Osagie, 1986	George & Arumghan, 1991
Palmitic	16:0	45	44	43
Stearic	18:0	5	4	4
Oleic	18:1	37	36	38
Linoleic	18:2	11	15	12
Others		2	1	3

Table 1.1: The Fatty Acid Composition of Triacylglycerol in Ripe Oil Palm Mesocarp and the Similarity in Composition of Malaysian, African and Indian Fruit

Oil content increases up until the start of fruit detachment (abscission) and after this the evidence for further oil accumulation is conflicting (Oo *et al*, 1986; Bafor & Osagie, 1986). However, free fatty acid content in the ripe fruit, which is low during incorporation into triacylglycerol, rises considerably in over-ripe detached fruit (Table 1.2).

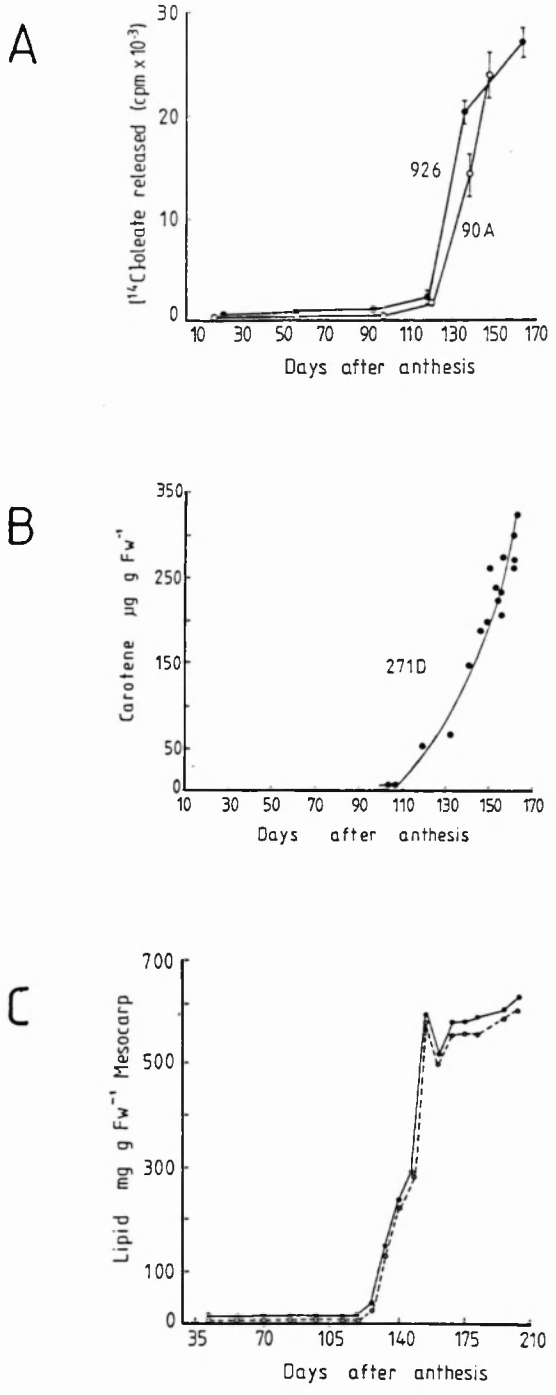


FIG. 1.5: LIPASE ACTIVITY, CAROTENOID BIOSYNTHESIS AND ACCUMULATION OF LIPID DURING RIPENING

A. Lipase activity in mesocarp of unripe, ripening and ripe fruit of two normal clones, 926 and 90A. (From Henderson & Osborne, 1991.)

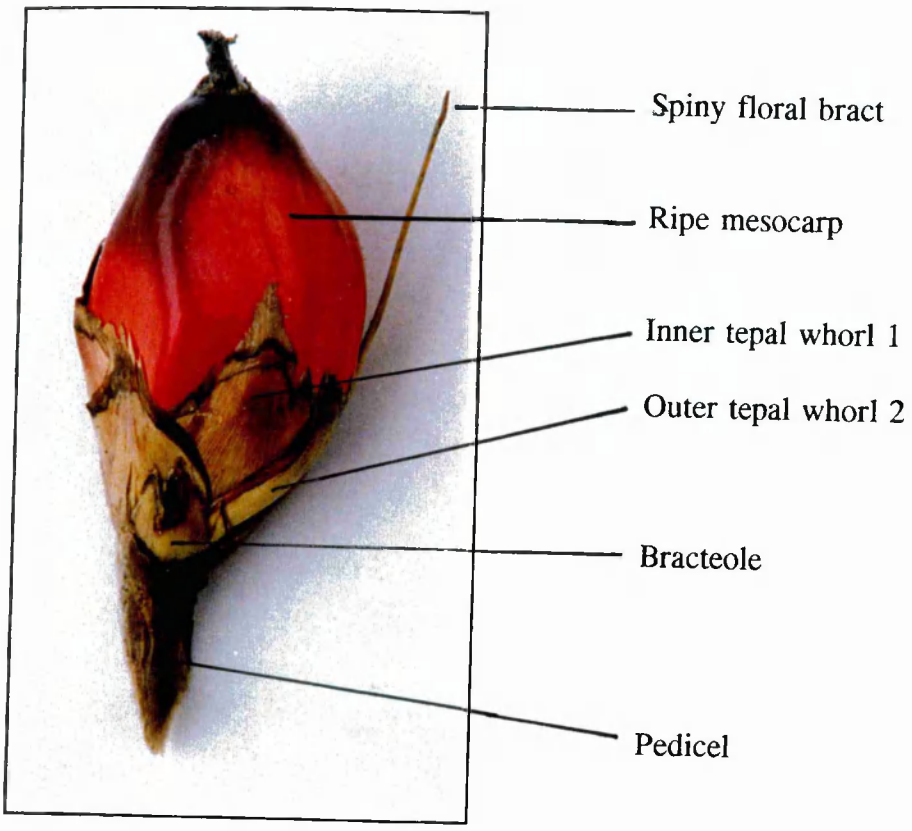
B. Carotenoid content (μg g Fw^{-1}) of mesocarp of unripe, ripening and ripe fruit of the normal clone 271D. (Extraction and determination of carotenoids by Ms A. Norville, see Appendix C.)

C. Accumulation of total lipid (●—●) and neutral lipid (o- - -o) in the mesocarp during development of Nigerian fruit: young unripe (42 daa) to overripe (180 daa-203 daa) fruit. (Re-drawn from Bafor & Osagie, 1986.)

Author(s)	% FREE FATTY ACID IN MESOCARP	
	Ripe fruit, not detached	Over-ripe fruit, detached
Dufrane & Berger (1957)	0.5%-1.2%	2.9%-5.0%
Coursey (1963)	0.1%-0.4%	-
Wood & Sing (1977)	0.33%	-
Esechie (1978)	0.6%	2.0%-4.0%
Bafor & Osagie (1988)	Not detected	3.6%
Hartley (1988)	0.1%-0.5%	-

Table 1.2: Fatty Acid Content (%) in Ripe Attached and Ripe Detached Fruit

The presence of a very active lipase had been long suspected in ripening fruit (Vanneck, 1947; Loncin & Jacobsberg, 1965). For some years it was known that the FFA content increased greatly in loosened or fallen ripe fruit, fruit that was bruised or cooled below 15°C, and fruit of cut bunches which were not processed for some days. This was attributed to lipolytic activity but attempts to identify an active endogenous lipase were not successful (Oo, 1981; Tombs & Stubbs, 1982). Lipolysis was attributed to microbial infection - the activity of the lipases of yeasts and moulds. However, in 1985 Abigor *et al* were able to partially purify an endogenous mesocarp lipase from the fatty layer of homogenates and, although this work was unknown to ourselves, WE ALSO DEMONSTRATED THE PRESENCE OF A VERY ACTIVE LIPASE by similar methods (Henderson & Osborne, 1991). In addition, WE SHOWED THAT THE RAPID INCREASE OF LIPASE ACTIVITY DEVELOPED AT THE SAME TIME AS THE FRUIT STARTED TO RIPEN, (Fig. 1.5A). THIS WAS CONFIRMED BY DETERMINING THE INCREASE IN CAROTENOIDS IN THE MESOCARP OF THE NORMAL CLONE 271D (Fig. 1.5B). Bafor & Osagie (1986) also showed that the deposition of triacylglycerol in Nigerian fruit mesocarp started to occur at about 133 daa (Fig. 1.5C). Thus, at the commencement of ripening, the increase in lipase activity coincided with the initiation of triacylglycerol synthesis. This lipase, then, would appear to be the ripening-associated expression of new gene activity. The same authors and others (see Table 1.2) observed that, in ripe unloosened fruit on the bunch, FFA never exceeded 1.2% and this may indicate that the lipase acts to hydrolyse triacylglycerol only after fruit abscission. It is possible that the enzyme may function in esterification/transesterification reactions in triacylglycerol biosynthesis, but at abscission there is a changed physiological state of the mesocarp. OUR ELECTRON MICROGRAPHS SUGGEST A LOSS OF MEMBRANE INTEGRITY OF ORGANELLES IN THE OIL-BEARING CELLS OF THE MESOCARP (Fig. 3.5, Section 3.1.2, pages 80 & 81A). This could result in the



A

B

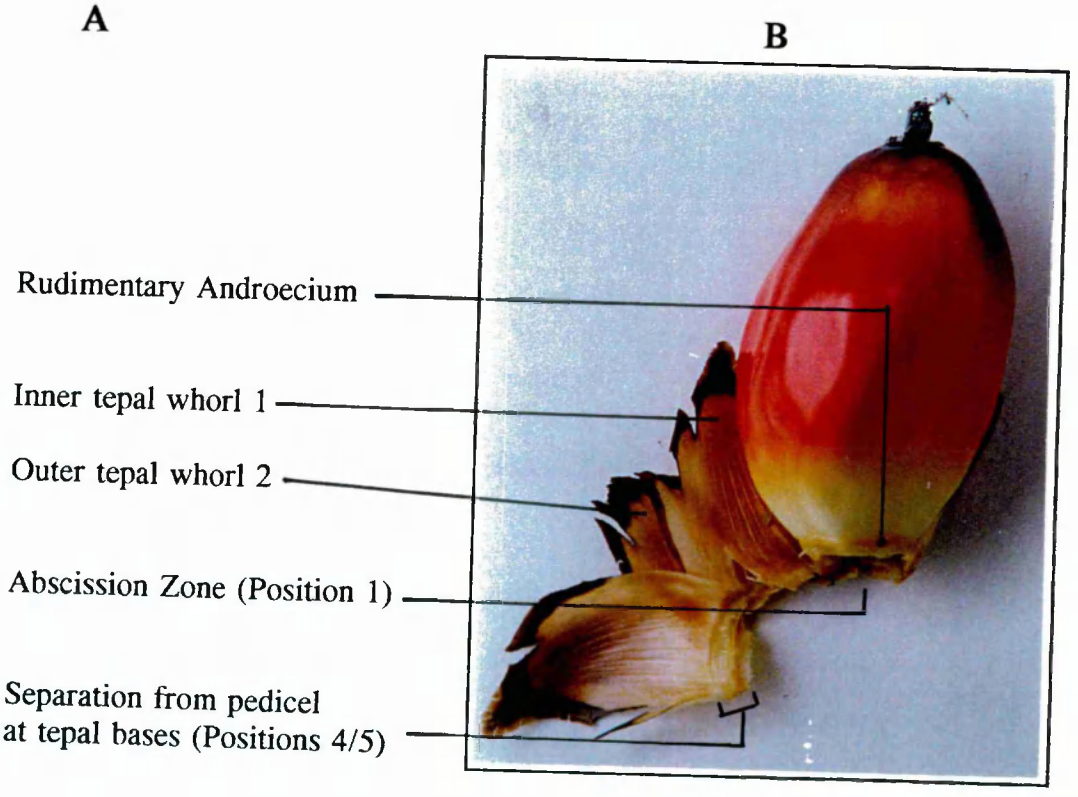


FIG. 1.6: THE OIL PALM FRUIT AND FLORAL APPENDAGES

A. A fruit removed from a spikelet with the spiny floral bract, bracteoles and tepals attached.

B. A fruit showing the position of the rudimentary androecium beneath the tepal whorls (this fruit has separated 1+4/5).

hydrolysis of the triacylglycerol. In a further study of the lipase by Sambanthamurthi *et al* (1995) 3.6% protein was found to be associated with the oil bodies in mesocarp cells. SDS-Page showed 7 protein bands and attempts were being made to raise antibodies to the proteins which might define more clearly the control of synthesis, exact localisation and nature of enzyme-substrate interaction.

During immature stages until about 120-130 daa the fruits are yellow-green with purple tops and between this time and full fruit ripeness (140-160 daa) the fruit colour changes, at first, becoming light yellow, then usually to a deep orange-red (Figs. 1.3 & 1.5B) although varietal differences in pigmentation are observed. Chlorophyll does not completely disappear in ripe fruits but from 120 daa onwards it decreases (Ikemefuna & Adamson, 1984). The relative concentrations of chlorophylls a and b in green fruit were reversed in ripe fruit. Palmitoleic and linolenic fatty acids - typical chloroplast fatty acids - were not detected in fruit after 120 daa and this may be an indication of a depression in photosynthetic activity (Bafor & Osagie, 1986). Whilst unripe fruit contain and synthesize some carotene, it is very low compared to that in ripe fruits. Ripe terminal or the exterior fruit on a bunch contain twice as much carotene as interior fruits (Hartley, 1988). Ikemefuna & Adamson (1984) report that in one variety of oil palm carotene content increased more than 300-fold from mature unripe to full ripeness. This massive accumulation of carotenes is suggested by the same authors to occur in chromoplasts formed *de novo* and as existing chloroplasts transform to chromoplasts.

1.3.2 Abscission of Ripe Oil Palm Fruit

Fruit ripening in a large bunch (e.g. banana, date, grape) is usually asynchronous and the oil palm bunch (Fig. 1.4) is no exception. Thomas *et al* (1971) studied ripening fruit in the whole bunch and observed different patterns of development for outer, middle and inner fruit (Fig. 1.4). Outer fruit are the largest in size weighing about 10-15g, they mature first and commence abscission about 20 days before central fruit. The formation of mesocarp oil in central fruit (which weigh about 3-7g) is slower than that of the outer fruit, and inner fruit (weighing only about 2g) do not mature at all as far as oil formation is concerned. The reason for the size differences of the fruit is due to the inflorescence architecture - there is simply insufficient space to allow full-size development of all but the outer fruit on each inflorescence. Once outer fruit start to separate (1-10 fallen fruit is the criterion by which bunches are harvested) abscission proceeds rapidly and the bunch

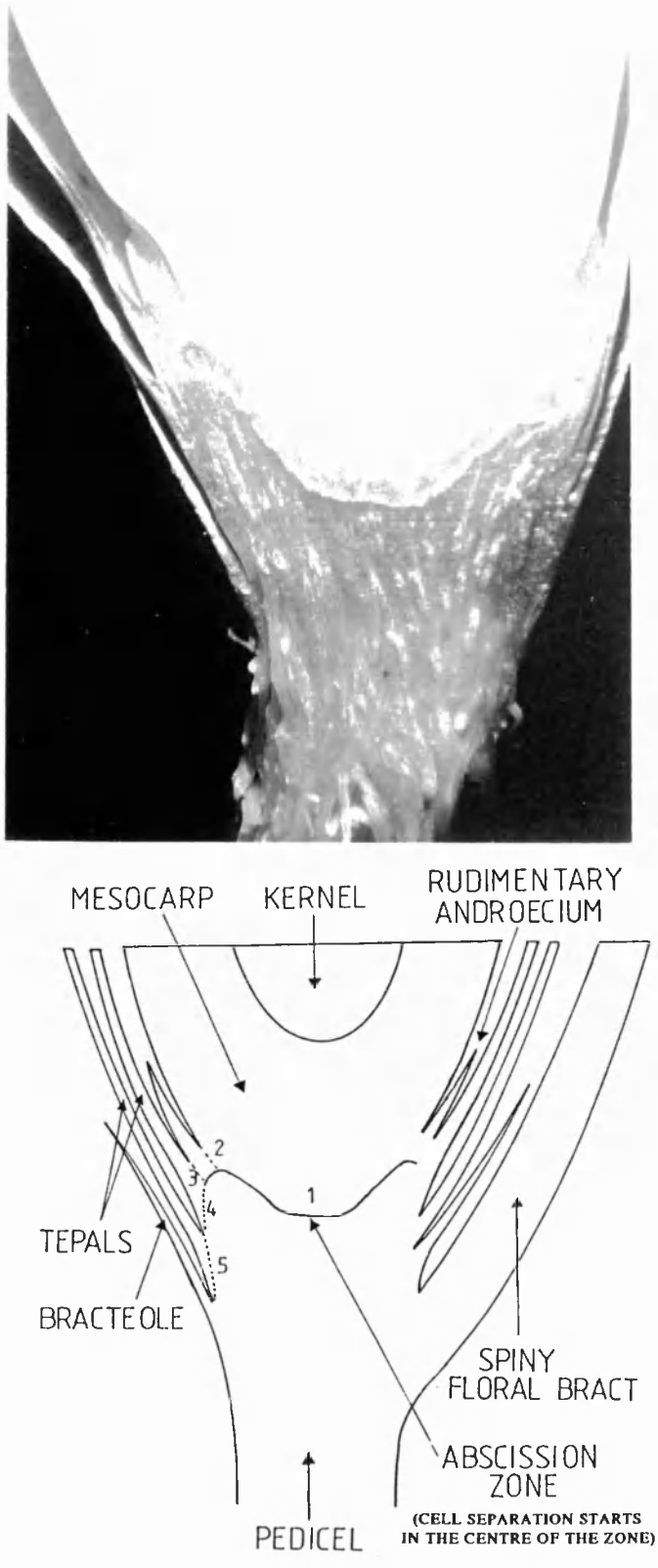


Fig. 1.7: **THE ABSCISSION REGION OF THE OIL PALM FRUIT**
(a longitudinal section)

The diagrammatic representation shows the position of cell separation events between the different tissue parts (the kernel and spiny floral bract are not seen in the photograph). (From: Henderson & Osborne, 1990.)

must be harvested to prevent the loss of these outer fruit since their triacylglycerol content is greater than that of the central or inner fruit.

The oil palm fruit *in situ* is encircled by a rudimentary androecium, two whorls of 3 tepals (6 tepals), two small bracteoles and a large, sharp, spiny floral bract (Fig. 1.6). The abscission zone of the fruit is differentiated even in pre-anthesis inflorescences. A recognisable plate of a few layers of small cells is present between the fruit and pedicel in the pre-anthesis fruitlets (Henderson & Osborne 1990). Ripe fruit that shed naturally from the bunch abscind along this same differentiated plate of cells to release a "naked" fruit. Cell separation begins in the centre of the zone and spreads to the fruit periphery (Fig. 1.7). This has been designated 1+2 separation (Figs. 1.7 & 1.8A). Harvested ripe bunches with unseparated fruit gradually shed their fruit over a period of 1 to 2 days but, unlike those shed from the palm, they are released with their subtending rudimentary androecium and some or all of the tepals still attached. This indicates that a further cell separation event has occurred between the pedicel and the tepals, designated as 1+4/5 separation (Figs. 1.7 & 1.10). It was of great interest to read in Stilliard's thesis of 1938 an account of the collection of palm fruit in Africa in the nineteenth century. His information was taken from an 1885 publication "On the Manufacture of Soaps and Candles" by W.L. Carpenter which related that after the African had harvested the bunches they were left in a heap for 7-10 days. After this time, the bunches were beaten and the fruits separated easily *"with their husks"*. These "husks", which are the tepals, were removed by hand or by rubbing the fruits together. THIS IS THE SAME TYPE OF SEPARATION THAT WE HAVE OBSERVED IN OUR LABORATORY IN OXFORD. THE FRUIT WHICH HAD NOT SEPARATED ON THE HARVESTED SPIKELETS GRADUALLY DID SO AFTER SOME DAYS WITH THEIR RUDIMENTARY ANDROECIUM AND TEPALS STILL ATTACHED (1+4/5 SEPARATION). This 4/5 separation of the tepals from their pedicel attachment occurs 1 to 2 days after a ripe fruit has separated (1+2) and is part of a post-fruit abscission process. The tepals are shed from the empty socket and remain as a separate and complete intact whorl (Fig. 1.8C). Similar separation of the tepal whorl is now known to occur after fruits have been shed naturally from the bunch in palms on the plantation (Section 2.2.1).

The specialised cells of the abscission zone, whilst not morphologically distinct except by size until just before abscission, can be visualised as distinct from the neighbouring fruit parenchyma or pedicel fibres by histochemical staining (Plate 3 in

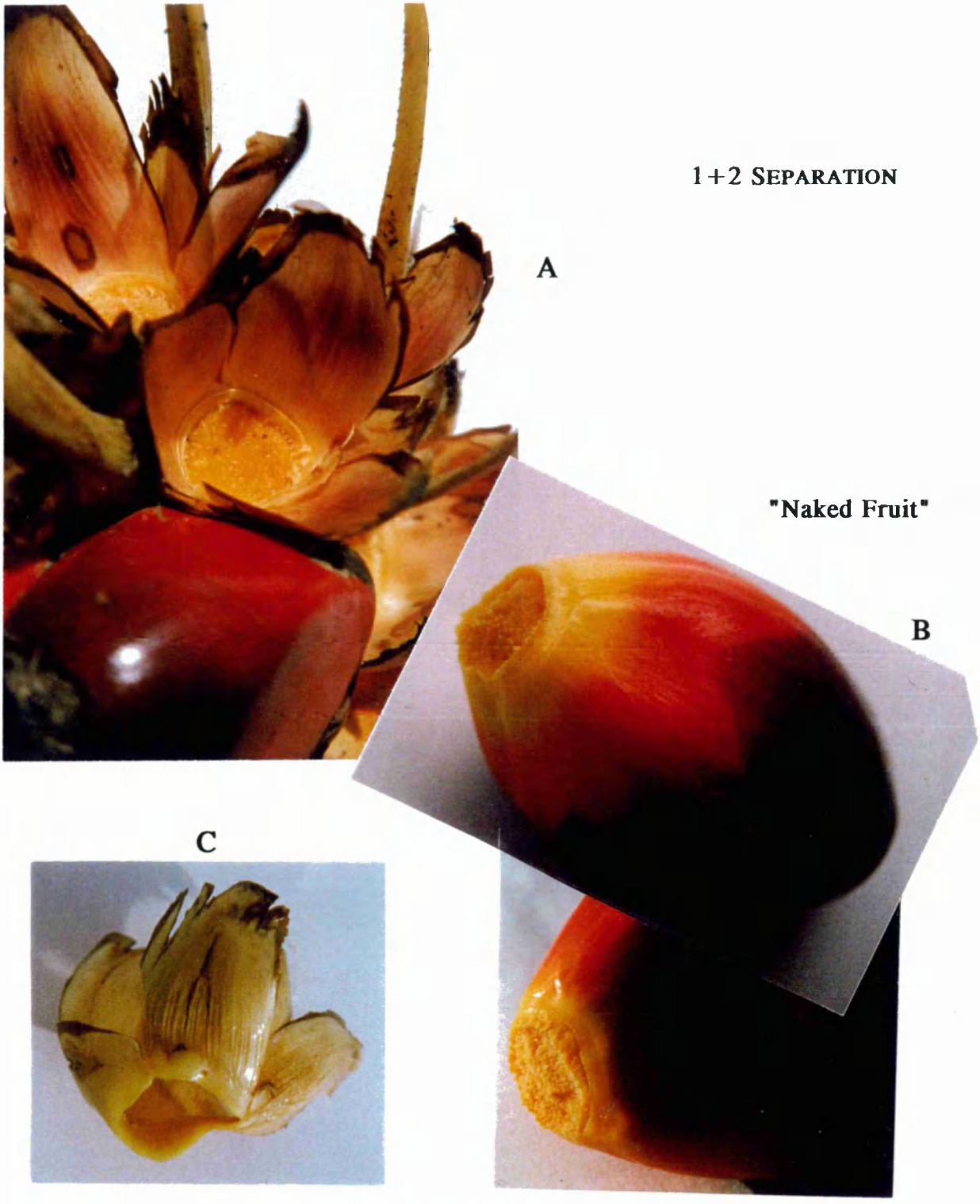


FIG. 1.8: 1+2 SEPARATION : NATURAL FRUIT SEPARATION

A. The RA remains within the cup of tepals after the fruit has been shed, encircling the pedicel side of the zone (PZ).

B. The fruit is shed "naked" (without the RA). Separation has occurred at Positions 1 and 2 (see Fig. 2.1, page 44A). Position 2 is seen at the base of the fruit, the "periphery", adjacent to the shiny, smooth epidermis.

C. The two whorls of tepals detach from the spikelet as a complete unit 24-48h after fruit abscission.

1+3 SEPARATION

A

B

FIG. 1.9: 1+3 SEPARATION

A. The RA remains attached to the fruit when it is shed. Separation has occurred at the outer face of the RA or Position 3 (see Fig. 2.1, page 44A). (Some fruit can detach with small pieces of RA adhering rather than the complete RA and natural separation from the palm occurs at both Positions 2 and 3.)

B. The pedicel side of the zone is shown surrounded by the tepal whorls (without the RA) on the spikelet (which will separate at Positions 4 and 5, as shown in Fig. 1.8C).

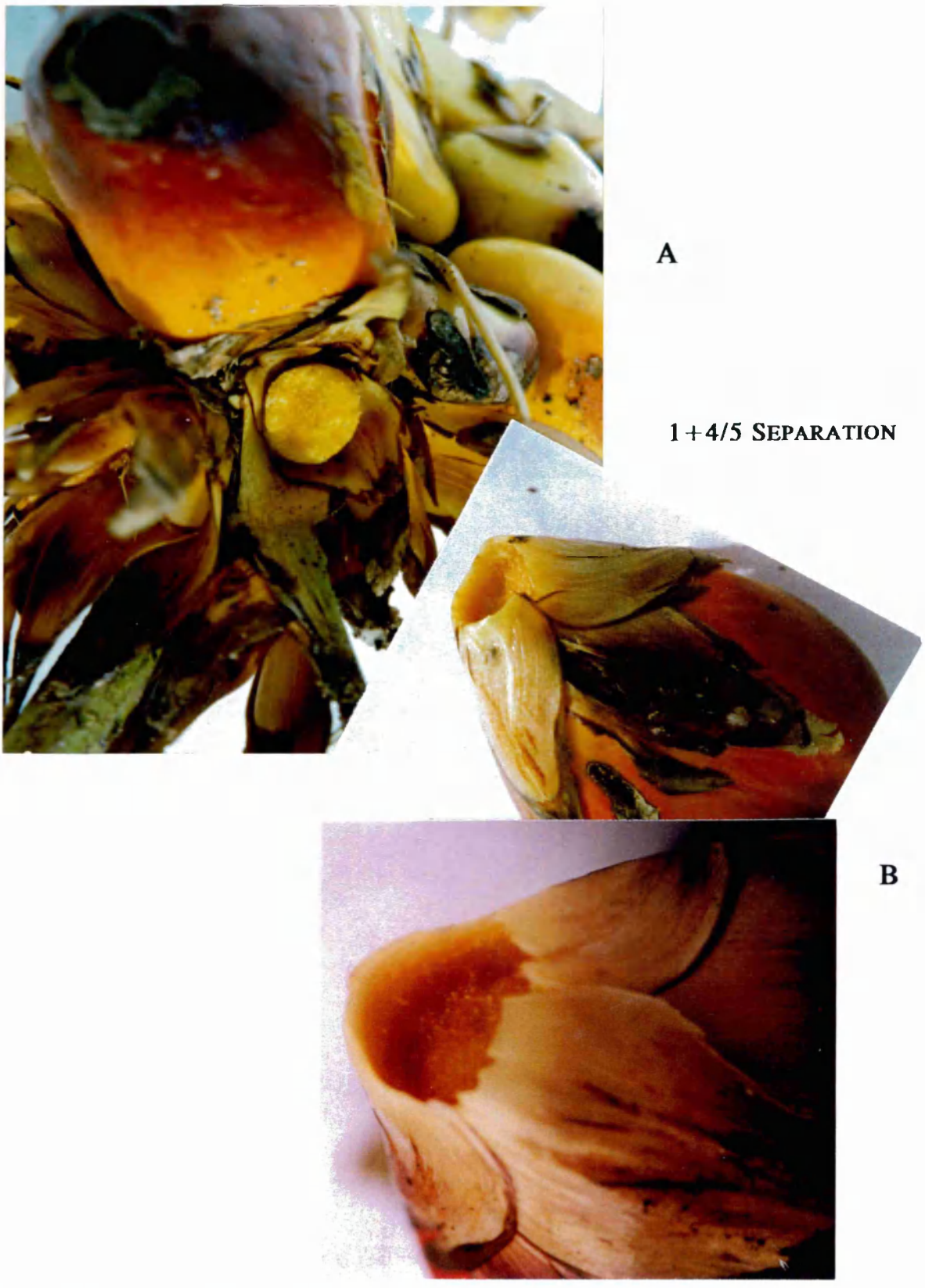


FIG. 1.10: 1+4/5 SEPARATION

A. The pedicel side of the zone (PZ) is surrounded by only the 2 bracteoles and spiny floral bract on the spikelet.
B. The fruit has separated with the 2 tepal whorls attached to the fruit. Separation has occurred at Positions 1, 4 and 5. Positions 2/3 have been bypassed (see Fig. 2.1, page 44A).

Henderson & Osborne, 1990). The cells have prominent nuclei with dense cytoplasm. After abscission, whether separation has occurred at 1+2, 1+3 or 1+4/5 positions (Figs. 1.7-1.10), scanning electron microscopy shows that both on the fruit side (FZ) and the pedicel side (PZ), the cells are still intact, that is, they have not been fractured during the separation process (Plate 5 in Henderson & Osborne, 1990, Appendix E).

Some proteins and enzymes have been identified in the oil palm abscission zone. Tissue blotting on to nitrocellulose membranes and detection of enzymes bound to the blot has revealed high activity of peroxidase and some ß-glucosidase activity in the zone (Spruce *et al*, 1987). Using a monoclonal antibody raised against abscission zone cells of *Sambucus nigra* (McManus *et al*, 1988), which preferentially recognised glycoproteins with a xylose/fucose oligosaccharide structure, high binding was shown to occur in the abscission zone of oil palm fruit at Position 1 only (Fig. 1.11; Osborne, 1992).

Fruit abscission has always been of interest to the oil palm industry because it has long been known that the free fatty acid content starts to increase in bunches that are shedding many fruit. A field study by Chan, Corley & Seth (1972) was conducted to assess the possibility of controlling fruit abscission with growth regulators - auxin, gibberellin and ethephon - with the hope of increasing fruit harvest yield. All three growth regulators appeared to delay abscission. The effect of ethephon was surprising because it usually enhances abscission; for example, in apple, cherry, plum, olive and grape where ethephon is used to loosen the fruit to facilitate mechanical harvesting (Lürssen, 1991). However, ethylene was suspected to enhance abscission in the oil palm despite this retardation by ethephon. Wuidart (1973) noted that removal by cutting 20 outer non-separating ripe fruit from a bunch resulted in faster abscission of the fruit around the areas from where the samples had been removed. This was indicative of a wound ethylene production and the subsequent enhancement of abscission. In addition, in 1977 Wood & Sing submitted a patent for an ethylene treatment of 1-3% which resulted in 99% separation of fruit from the harvested bunch within 15-20 hours.

1.3.3 Why Study Fruit Ripening and Abscission in the Oil Palm?

The position of palm oil in the world market has greatly improved due to the vast amount of research into the nutritional advantages of the oil, its versatility in substituting for other oils and its end-use applications. At the present time, palm oil is expected to

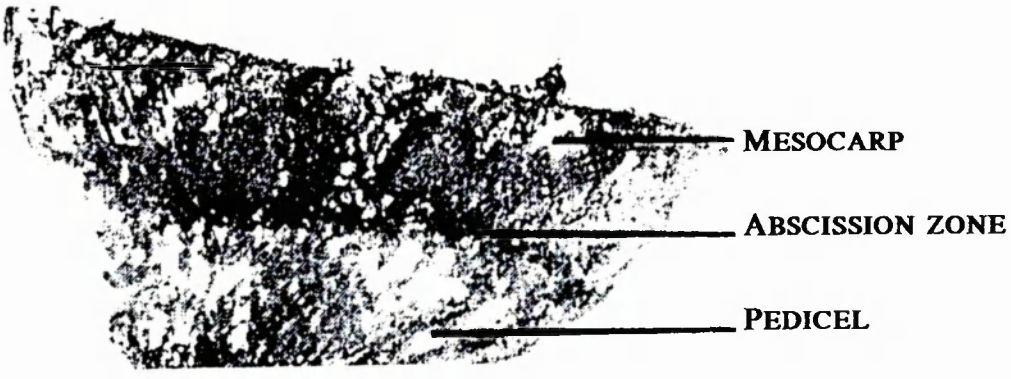


FIG. 1.11: PREFERENTIAL BINDING OF THE YZ 1/2.23 ANTIBODY TO THE ABSCISSION ZONE (Reproduced from Osborne, 1992.)

Longitudinal section of oil palm fruit shows the abscission zone. The blot was probed with the monoclonal antibody YZ1/2.23 to the heptasaccharide epitope Manα3 (Manβ6) (Xylβ2) Manβ4 GlcNAcβ4 (Fucα3) GlcNAc stained with immunogold-silver enhancement. Note the preferential staining of the abscission zone tissue.

"again be able to step into the breach and cover the bulk of world import demand" (Oil World Quarterly, 1996), since it has been predicted that there will be a large deficit in the world production of fats and oils in 1996/97 due mainly to a decline in production of the seed oils.

The importance of obtaining the maximum yield of oil possible from the fruit is therefore clear. The oil palm industry in Malaysia has been able to increase yields with improved breeding programmes and more efficient harvesting and processing. But despite this progress, a persistent problem, with no easy solution, is that of the non-synchronous ripening of fruit bunches. This makes choosing the best time to harvest a bunch to maximise oil yield, whilst keeping costs reasonable, a highly subjective process. The economically valuable triacylglycerols start to be hydrolysed to free fatty acid when the fruit loosen, that is, at the time of cell separation at the fruit's abscission zone (Esechie, 1978). It has been shown that the bunch has its maximum amount of oil at a stage of ripeness that is related to about 4-6 shed fruit per kilogram of bunch weight (Wuidart, 1973; Wood, 1981). Under-ripe bunches will not have reached their maximum oil yield, whilst over-ripe fruit on fully ripe bunches start to shed rapidly. It takes harvesters a long time to collect individual fallen fruit and many are lost on the way to the factory (collecting fallen fruit increases the cost of harvesting and their higher free fatty acid content decreases overall quality). So the common field criterion for harvesting of 1 to 10 fruit which have fallen to the ground (Somasundram *et al*, 1994) is really a very crude measure for determining time for bunch harvest considering the enormous economic importance of the crop.

Molecular biological technologies are now being employed to overcome commercial problems of fruit ripening in other species. For example, the FLAVR SAVR™ tomato is the first genetically engineered whole-food now to be available. It was developed so that fresh market tomatoes can be vine-ripened for enhanced flavour and have a longer shelf-life (Kramer & Redenbaugh, 1994, however, also see Errington *et al*, 1997). The polygalacturonase (PG) gene was isolated from ripe tomato fruit and reintroduced into plants in the antisense orientation. This reduces expression of the PG gene and results in fruit with improved storage and processing characteristics. Similar technology is being applied to the apple introducing antisense to the ACC synthase gene to decrease the post-harvest ethylene evolution. This suppression of "ripening hormone" is again expected to promote a longer shelf-life (Prof. D. James, personal communication, 1996). In addition,

the first transgenic oilseed crop with a modified seed composition - a lauric oil rapeseed - was cultivated for commercial use in 1995 and this technology is directly applicable to the oil palm (Murphy, 1996). No studies as yet have addressed a molecular modification of the shedding processes of the ripe fruit.

Since the elucidation of the biochemistry of ripening and abscission of oil palm fruit is a matter of such importance to the industry, knowledge of the control and enzymatic regulation of cell separation processes and triacylglycerol synthesis and hydrolysis is fundamental as a prelude to molecular modification. Although a long-term project, there would be great potential in successful antisense technology in the oil palm industry. For example, an extension of triacylglycerol synthesis and/or a reduction in lipid hydrolysis at ripeness; or a delay/prevention/synchronisation of abscission could all result in an increased oil yield per bunch and per palm.

1.4 THE RESEARCH TO BE DESCRIBED IN THIS THESIS

The question about the actual role of ethylene in oil palm fruit ripening and abscission and the potential value of ripening and abscission control laid the basis for this research and, initially, was commissioned by Dr. R.H.V. Corley of Unilever Plantations Research.

This thesis seeks to lay the foundation of the biochemistry of fruit ripening and abscission in the oil palm. It shows that the plant hormone ethylene and the induction of cell wall hydrolases in the mesocarp do **not** play a major role in the ripening of the oil palm fruit in contrast with many other fruits (e.g. tomato, avocado). A significant and measurable ethylene production in the fruit occurs only at abscission with an increased tissue-specific cell wall hydrolase activity in the mesocarp and the zone. Fruit separation is described as a two-phase process that is controlled by a number of specific enzymes and isoenzymes induced as part of the abscission process - in particular, cellulase and polygalacturonase.

To help elucidate some of these mechanisms, non-abscinding mutants and palms with aberrant abscission behaviour were sought (and discovered) in the Malaysian plantations. Ripening and abscission events have been followed in fruits of these mutant and normal clonal palms. Finally, proposals are made on how this information could be used for the manipulation of shedding and hence for the improvement of palm oil yields.

CHAPTER 2
MATERIALS AND METHODS

2.1 CHEMICALS, BIOCHEMICALS AND PREPARATION OF SOLUTIONS

All chemicals used were of analytical grade.

2.1.1 Chemicals and Biochemicals obtained from BDH, Poole, Dorset.

Acetic acid (glacial), acetone, ammonium sulphate, boric acid, calcium chloride .$2H_20$, chloroform, citric acid, Coomassie Brilliant Blue (G250, CI42655), D-glucose, disodium tetraborate (borax), galacturonic acid, glycine, HCl (35%), hydrogen peroxide (6%), magnesium sulphate.$7H_2O$, methylated spirit, nickel chloride, orthophosphoric acid, petroleum ether (60-80°C), pH standards 4.0, 7.0, 9.2 buffer tablets, PAGE Blue 83 (R250, CI42660) phloroglucinol, polyethylene glycol 6000, polyvinylpyrrolidone (MW 44,000), potassium chloride, potassium dihydrogen phosphate, potassium hydroxide, sand acid washed: 400-100 mesh (140μm-80μm), sodium acetate, sodium azide, sodium bicarbonate, sodium carbonate.$10H_2O$, sodium chloride, sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium hydroxide, sucrose, sulphamic acid, 5-sulphosalicylic acid, sulphuric acid, trichloroacetic acid, trisodium citrate, Tween 20, universal indicator.

2.1.2 Chemicals and Biochemicals obtained from Sigma, Poole, Dorset.

Albumin (bovine serum), APMSF (4-amidinophenylmethane sulphonyl fluoride), basic fuchsin, 3,3′-diaminobenzidine, bromthymol blue (3′3″-dibromothymol sulfone-phthalein), dithiothreitol, ethylenediaminetetraacetic acid disodium salt, Goat Anti-Rabbit IgG peroxidase conjugate, L-histidine, *m*-hydroxydiphenyl, IEF-Mix 3.5-9.3, leupeptin, 2-mercaptoethanol, methyl-α-D-mannopyranoside, neocuproine hydrochloride, α/ß-*p*-nitrophenyl-glycosides, *p*-nitrophenol, Nonidet P-40, ovalbumin chicken egg, pectinase (*Aspergillus niger),* pectinesterase (tomato), *o*-phthaladehyde, phenylmethyl sulfonyl fluoride, polyvinylpolypyrrolidone (insoluble), protease *Streptomyces griseus* Type XIV, Rabbit Anti-Rat IgG peroxidase conjugate, ruthenium red, sodium sulphide, sodium sulphamate, sodium dodecyl sulphate, triethanolamine hydrochloride (2,2′2″-nitrilotriethanol), 2,3,5-triphenyltetrazolium chloride (tetrazolium red), Trizma base (tris[hydroxymethyl]aminomethane).

2.1.3 Polysaccharides obtained from Sigma, Poole, Dorset

The following details of the polysaccharide substrates used in enzyme analyses are given:

- Chitin (poly-1,4-ß-N-acetyl-D-glucosamine from crab shells).
- Gum arabic (from *Acacia* tree; believed to be a branched polymer of galactose rhamnose, arabinose and glucuronic acid; MW ~250,000) Note: Aldrich Chemical Co. supplies galactan substrate derived from gum arabic.
- Laminarin (from *Laminaria digitata*, 95% ß-1,3-polyglucose; may contain small amount of ß-1,6 as inter-residue linkages or as branch points, traces of mannitol as end groups and pentose; MW 8,600).
- Lichenin (from *Cetraria islandica*; linear glucose polysaccharide, two ß-1,4 and one ß-1,3 residues).
- Locust bean gum (from seeds of *Ceratonia siliqua*; believed to be a straight chain polymer of mannose with one galactose branch on every fourth mannose; MW ~310,000).
- Pectin (from *Citrus*, partially methoxylated polygalacturonic acid; DM minimum 7%; galacturonic acid 89%; MW ~23-71,000).
- Polygalacturonic acid potassium salt (from orange; DM $<1.0\%$; MW 15-40,000, av. 28,000).
- Xylan (from birchwood, poly-ß-D-1,4-xylopyranose; $>90\%$ xylose residues).

2.1.4 Chemicals and Biochemicals obtained from other companies.

- Carboxymethyl cellulose (CMC) was obtained from Hercules Incorporated, Cellulose & Protein Products Department, Delaware.
- Pachyman (from *Poria cocos*, ß-1,3-linked glucose polymer containing 95% anhydroglucose, MW (3000)n) was obtained from Calbiochem, Nottingham.
- Pectin samples of acid-washed high (HM) and low (LM) methylation were a gift from Citrus Colloids Limited, Hereford (Table 2.1).

Pectin Sample	Degree of Methylation (DM) %	Galacturonic Acid %	Molecular Weight
Lime HM	70.8	85.7	546,000
Lime LM	31.8	87.4	425,000
Apple HM	72.0	75.4	630,000
Apple LM	31.2	76.3	379,000
Grapefruit HM	72.4	78.4	972,000
Grapefruit LM	30.6	87.3	592,000

Table 2.1: Details of the Pectins donated by Citrus Colloids Limited

2-cyanoacetamide was obtained from Aldrich Chemical Company, Gillingham, Dorset; α-aminoxyacetic acid hydrochloride from Calbiochem, Nottingham; Methylene blue (CI52015) from Fisons, Loughborough, Leics. Disodium tetraborate, sodium hydroxide and trichloroacetic acid were Prolabo chemicals from Philip Harris, Park Royal, London.

2.1.5 Preparation of Buffers and all other Solutions

The water used to prepare solutions was equivalent to double-distilled and purified using an Elgastat Option 3 Ultra high quality (UHQ) water purifier. This system utilises reverse osmosis, adsorption, deionisation and ultraviolet irradiation to achieve water purity higher than that of distilled water, that is, a conductivity of $\sigma < 1$ $\mu S.cm^{-1}$ at 25°C. No ion or free metal in UHQ water has a concentration greater than $0.2\mu g\ l^{-1}$.

All pH adjustments were carried out at room temperature using a Whatman AS 507 pH/conductivity meter, calibrated with pH 7.0 and either pH 4.0 or pH 9.2 buffer standards (BDH).

2.2 PLANT MATERIAL

Freshly-harvested spikelets of clonal material from Malaysia were shipped air freight, in loose cotton bags, enclosed in perforated polythene and boxed in a perforated cardboard container. Spikelets were received in Oxford 24-30h after excision from the parent palm and were then used as experimental material.

The temperature of fruits during transit did not fall below 12°C, nor did it rise above that experienced by fruit in the field (max 40°C). It was confirmed that the cargo

hold temperature was the same as the cabin temperature of between 13°C and 18°C. Often tropical fruits like the oil palm are sensitive to chilling. On a few occasions, in winter, the cargo had been exposed to the cold, that is below 10°C, for a few hours when off-loaded at Heathrow during which time the fruit became chilled. In these circumstances, after a few hours at room temperature the fruit mesocarp turns a browny-orange/yellow depending on stage of ripeness and it is quite evident that the fruit have been chilled. These fruit were not used in experiments.

Each spikelet was of a known number of days from anthesis and consisted of some 10-25 fruits. Abscission of ripe fruit in the field for clone 271D was reported by Pamol, Malaysia, to be between 140-150 daa (average 144 daa) but since a few deliveries of non-separating ripe fruit of about 150 daa have been received in the laboratory, we determined and recorded the stage of fruit abscission on arrival (Section 2.2.2). In fact, abscission in ripe normal fruit can vary between 128 daa to 180 daa (Dr H. Corley, personal communication). Fruit is described as ripe, ripening or unripe because seasonal differences in rates of fruit maturation do not permit direct comparisons of data at the same days after anthesis. Nomenclature for the different floral parts is based on that of van Heel, Breure & Menendez (1987).

The clone 271D was used for the majority of the experiments presented in this thesis. This cloned material was from "tenera" fruit which has a medium shell thickness (Fig iiB, Preface, page ii) of the inner kernel (a single gene controls shell thickness; tenera is the heterozygote from a cross between dura (thick shell) and pisifera (thin shell)). Clones 90A, 926, 476G have also been used and no difference in abscission behaviour between clones was observed. Four palms which did not shed their fruit were discovered. One was in the Kluang plantation in Malaysia (DxP5) and three in the Sabah plantation in Indonesia. Fruit spikelets from the Sabah plantation were received in Oxford about 48h after excision from the parent palm. These required an extra day to dispatch from Indonesia via Singapore to Heathrow unlike fruit from the Kluang plantation (24-30h). On several occasions the complete fruit bunch of the Kluang mutant palm, rather than individual spikelets, was dispatched to the laboratory in Oxford. Only a limited number of investigations were carried out on the three Sabah non-abscinding mutants and the results presented in this thesis relate mainly to the normal clone 271D and the non-abscinding Kluang mutant.

The amount of fruit tissue, other than the mesocarp, that was obtainable from each delivery was quite small in relation to the total weight received. From 30 spikelets (~3-4 kg) about 3g of scraped zone cells or excised unseparated abscission zones and 5g of tepal bases could be collected. However, for some experiments, it was not possible to obtain any tissue sample sufficient for enzyme analysis. These were used in experiments for ethylene production and the subsequent assessment of abscission; electron microscopy; histochemistry; uronic acid determination; experiments using tissue slices, and so forth.

A fresh fruit shipment from Malaysia entailed driving it to the airport at Singapore, air freight to the U.K., customs clearance and courier delivery to Oxford. Each delivery cost in the region of £500 and, since about 200 shipments of oil palm have been received, this has represented a sizeable investment on the part of Unilever in this research.

2.2.1 Abscission of Fruit from the Palm on the Plantation

Pamol Plantations, Kluang, Malaysia, were asked to check the location of the rudimentary androecium after the separation which had occurred in the naturally-abscinded, fallen fruit found around the base of the palms. Their results are presented in Table 2.2.

		RUDIMENTARY ANDROECIUM		
	Total fruit observed	**Attached to fruit (Shed 1+3)**	**Partly attached to fruit (Shed 1+2/3)**	**Not attached to fruit (Shed 1+2)**
90A	100	0	1	99
Seedling	205	17 (8%)	26 (13%)	162 (79%)
271D	109	13 (12%)	41 (38%)	55 (50%)

Table 2.2: Fruit which Abscind Naturally from the Palm were Examined (on the plantation) for the Position of Separation: 1+2, 1+2/3 or 1+3 (See Figs 1.8 and 1.9)

These results show that on the plantations the natural separation of ripe fruit is generally without its rudimentary androecium ("naked") or with small patches only of rudimentary androecium (RA) adhering.

As discussed in Section 1.3.2, after fruit abscission the RA remains attached to the inner tepal whorl on the spikelet and this type of separation is "1+2" (Fig. 1.8, page 35A). Some fruit also separate with the RA attached to the fruit, and this is designated

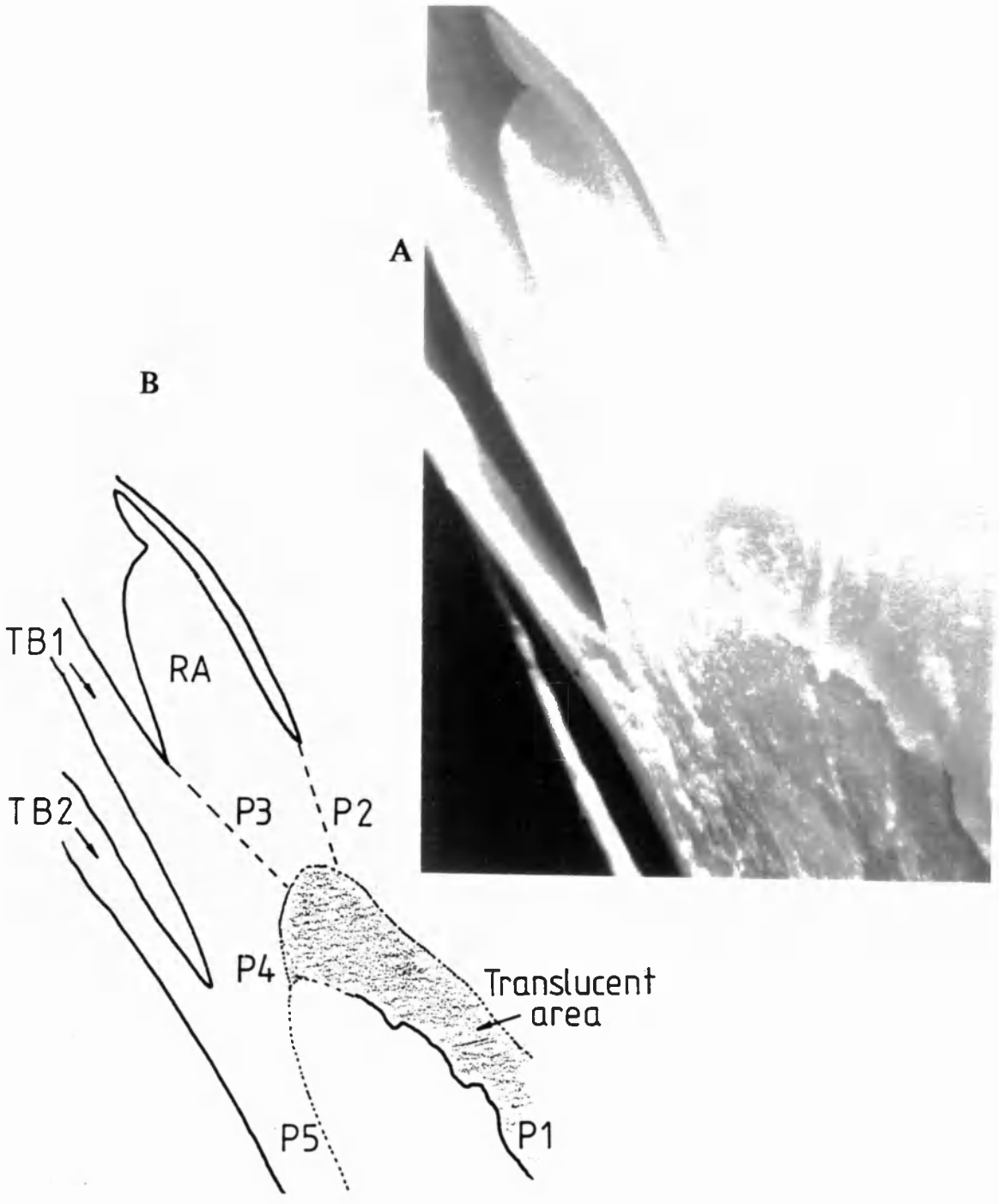


FIG. 2.1: AN ENLARGED VIEW OF THE ABSCISSION REGION AT THE PERIPHERY OF THE FRUIT

A. Separation at Position 1 (P1) has occurred and the tissue directly above is translucent. No separation is occurring at Positions 2 or 3 (P2, P3) below the rudimentary androecium (RA). Positions 4 and 5 (P4, P5) involve separation at the base of the tepals 1 and 2 (TB1, TB2).

B. Diagrammatic representation showing the positions of separation.

"1+3" (Fig. 1.9, page 35). Separated fruits with small patches of RA are "1+2/3". This indicates the critical participation of cells at Position 1, the zone itself, and, additionally, by cells at the base of the rudimentary androecium (Positions 2 or 3) during the abscission process (Fig. 2.1).

After the fruit is shed, the tepals remain attached to the spike. The field staff were asked if they had ever seen ripe fallen fruit with attached tepals, that is, fruit which had separated from the fruit bunch in Positions 1+4/5 (Fig. 1.10, page 36A). They reported that this had **not** been observed for normal fruit. However, the separation of the tepals, **after** fruit abscission, from spikelets of the fruit bunch *in situ* was assessed as follows:

> 159 cups of tepals remaining after fruit abscission on spikelets of the fruit bunch on the palm were tested for removal of tepals easily by hand. The first whorl of tepals in 121 cups of tepals separated the same day as fruit were shed and detached easily by hand. The next day, in these 121 cups, the second whorl of tepals, and the two whorls of the remaining 38 cups, detached.

This showed that separation at Position 4/5 to release the whorls of tepals occurs as a post-fruit abscission event in the field from the fruit bunch *in situ*; it took place only **after** 1+2, 1+2/3 or 1+3 separation had occurred (Fig. 2.2A). Then, only the large spiny bract and the two small bracteoles are left upon the spikelet (Fig. 2.2B).

2.2.2 Assessment of Abscission after Harvesting of Spikelets

On arrival in the laboratory **ripe** fruit could be categorised into the following stages of separation:

- Some ripe fruit very close to abscission when harvested in Malaysia separate in transit. These would be closest to the natural abscission from the palm. The type of separation was always either 1+2, 1+2/3 or 1+3.
- Ripe fruit not as close to natural abscission, but which had separated at Position 1, were just completing separation at Positions 2 or 3. These fruit would come off without any force and detach easily with a gentle "wiggle".
- Ripe fruit that had separated at Position 1 but not at Position 2 or 3. These fruit would separate with some pressure and a little "snapping" noise was heard

A

B

FIG. 2.2: SEPARATION OF THE TEPAL WHORL FROM THE SPIKELET

A. The tepal unit comprising of tepal whorls 1 and 2 after the fruit is shed (1+2 or 1+3). The two whorls remaining on the spikelet gradually separate from the pedicel but not from each other and are shed as a complete unit. They also can be removed easily by hand as a complete ring of tepals.

B. The large spiny floral bract and two small bracteoles remain on the spikelet. These are never shed from the spikelet either on the palm or in the laboratory.

indicating complete cell separation at 2/3 had not occurred and this position was being broken.

- Ripe fruit which had not separated. To determine if separation of the fruit had taken place at Position 1 in fruit that was still attached to the spike, fruit were removed from the spikelet. A transverse cut (with a Stanley knife) was made below the kernel of the fruit. The lower part of the fruit was then cut in half longitudinally with a single-edged blade (Fig. 2.9, page 71A) and gentle pressure exerted on the fruit side and on the pedicel side of the zone. Non-abscinding fruit will not separate under these circumstances, even when great force is applied. Immediately before separation starts, the zone becomes translucent and then separation occurs first in the centre of the zone. A fruit that is already partly separated at Position 1 then readily dissociates one side from the other with gentle pressure and this is easily seen by eye.

Tepal separation was similar to that in spikelets of the fruit bunch *in situ* on the plantations. After fruit had separated and shed, the whorl of tepals gradually separate from the sides of the pedicel usually within 48h if the spikelet is maintained at 75% relative humidity. This separation involved Positions 4 and 5 (Fig. 2.2).

On arrival in the laboratory, **unripe**, **ripening** or even **ripe** fruit spikelets that were not close to abscission when harvested in Malaysia would separate within several days:

- During the following 24-48h separation occurred at Position 1 and Positions 4 and 5 and fruit are released with subtending RA and tepal whorls 1 and 2 attached. As discussed in Section 1.3.2 and shown in Fig. 1.10 (page 36A) this was called 1+4/5 separation. These fruit undergo the same changes in the zone but it takes a longer time to achieve separation (depending on the stage of ripeness). Separation usually did not occur at all at Positions 2 or 3 in these fruit. It was by-passed; rather, in harvested spikelets, the separation that occurred at Positions 1 and 4/5 was simultaneous although it would have been sequential in the natural abscission of fully ripe fruit *in situ* in the field (Section 2.2.1).

2.2.3 Time Course of Fruit Abscission at Position 1

In experiments to determine the ethylene production of ripe fruit, analyses were made on their arrival in the laboratory and of their stage of separation at this time and for

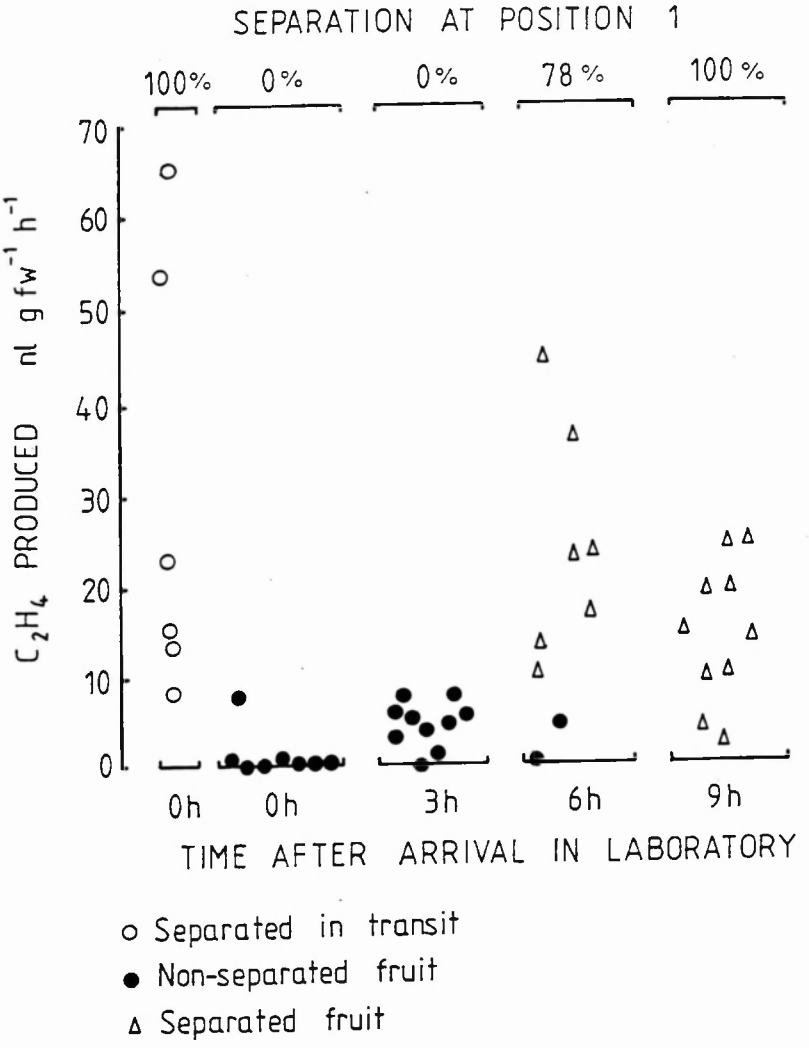


FIG. 2.3: ETHYLENE PRODUCTION IN RIPE FRUIT ON ARRIVAL IN THE LABORATORY (From Henderson & Osborne, 1994)

Six fruit had separated in transit, the remaining fruit were unseparated and their ethylene production was monitored during the following 9 hours. The fruit were then examined by longitudinal section for separation at Position 1.

the next 24h. (Fig. 2.3; reproduced from Henderson & Osborne, 1994). Ripe fruits which had not separated on arrival produced barely detectable levels of ethylene but within 6-9h ethylene production had increased coincident with the onset of Position 1 separation. Ripe fruit which had shed in transit were also producing ethylene. This indicates that the ethylene production of the fruit appears to provide a signal for cell separation at the abscission zone, and that in ripe fruit it can rise to very high values in a short space of time.

In ripe fruit which had not separated, when cut in half longitudinally, the zone was observed to be either not translucent or translucent. Only the fruit with translucent zones were producing some ethylene. Fruits with non-translucent zones were essentially non-producing. The time taken for a ripe zone to undergo the change from not translucent at all to complete separation at Position 1 could be as little as 6-9 hours. Thus, when the fruit arrived at the laboratory it was important to assess with accuracy the stage of fruit separation and to collect tissue samples as soon as possible.

2.3 COLLECTION OF FRUIT TISSUE FOR ENZYME ANALYSIS

Initially, tissue samples were collected into cooled glass vials on ice 24-48h after arrival. Enzyme extracts of the tissues were prepared (tepal base 1, mesocarp and abscission zone) and from these were obtained ammonium sulphate protein preparations (Section 2.4). Analysis of these protein preparations indicated that certain enzyme activities increased in ripe separated fruit. In addition, initiation of ethylene synthesis in fruit correlated with Position 1 separation (Section 2.2.3). Since these enzyme changes occurred within a very short time, a more rapid collection method was adopted for accurately pinpointing the appearance of new enzyme activities.

When the fruit arrived in the laboratory, the fruit were assessed for their type of abscission (1+2, 1+2/3, 1+3, 1+4/5) or how close to abscission they were (Position 1 only, translucent zone, not translucent) as described in Section 2.2.2. These were designated as 0h samples and tissue was collected from them. This was the earliest time we could obtain tissue samples after delivery and it represented a post-harvest age from the parent palm of about 24-30h. The samples were collected directly into glass vials standing in a mortar placed in a polystyrene container. The polystyrene container contained liquid nitrogen and so the samples were frozen immediately. They were then stored at -20°C.

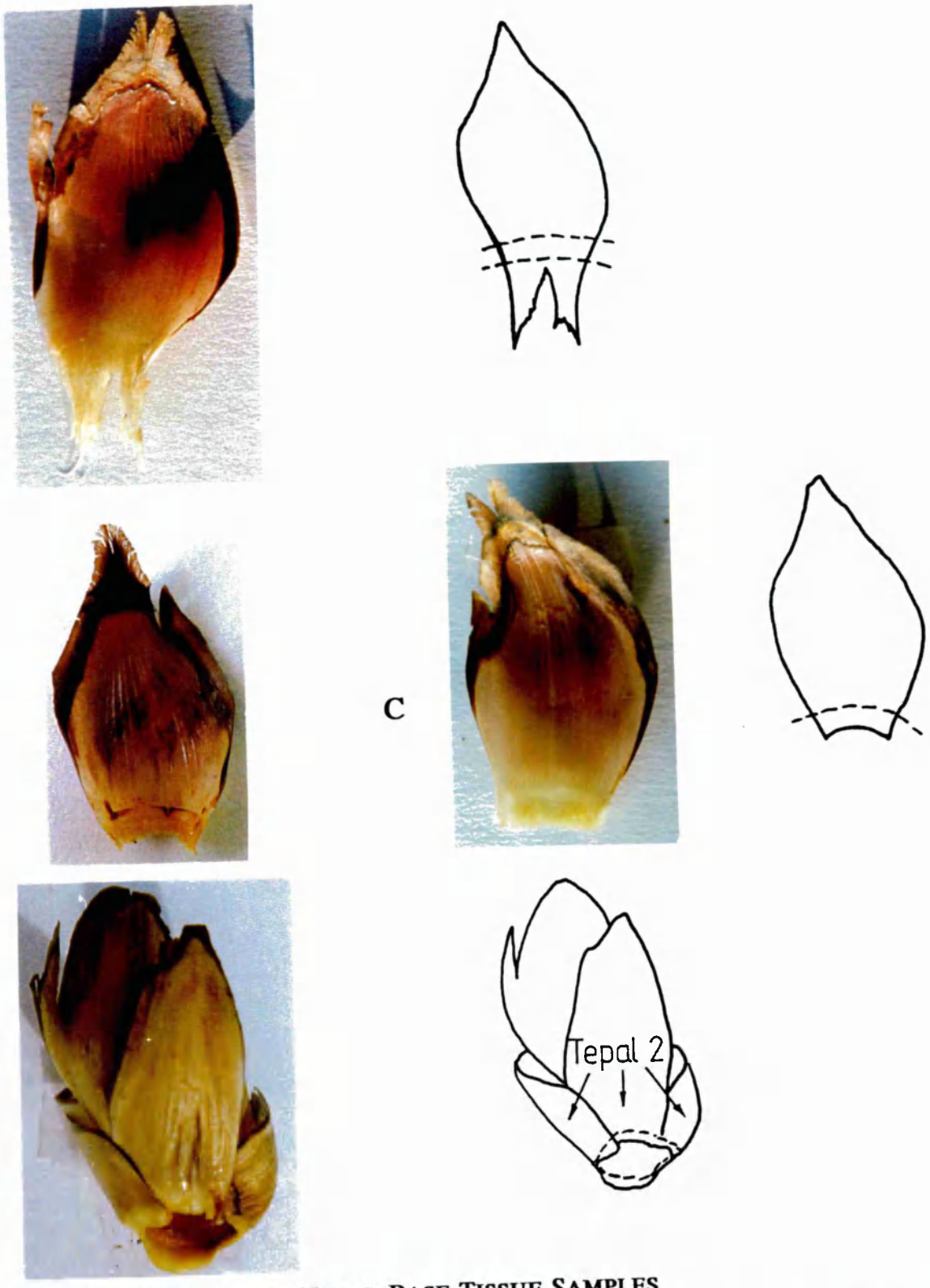


FIG. 2.4: PREPARATION OF TEPAL BASE TISSUE SAMPLES

A. Tepal 1 from an unseparated fruit. The tepal itself above the area of presumptive separation and the fibres below were cut away to prepare an unseparated "tepal base" sample, as shown in the diagram.

B. Tepal 1 from a 1+2 separated fruit. The tepal base below the rudimentary androecium was removed for the tissue sample (as shown in the diagram below the broken line).

C. Tepal 1 from a 1+3 separated fruit. Although the RA remains with the fruit, it causes an indentation in the tepal and the tissue below this was removed (as shown in the diagram).

D. The tepal whorls after separation from the pedicel. The outer whorl of 3 tepals (tepal 2) was peeled from the inner whorl (tepal 1) and the tissue from the base of tepal 2 was removed (as shown in the diagram below the broken line).

Tissue samples of mesocarp, separated zone cells (FZ, PZ), the fruit periphery, unseparated zone, rudimentary androecium (RA) and tepal bases, were collected from unripe and ripe fruit. Thin slices of mesocarp were taken from the middle of the fruit after its exocarp (epidermis) had been removed. Separated ripe zones, the fruit side (FZ) and pedicel side (PZ), were scraped with a single-edge dissecting needle. The periphery of the separated zone was dissected from the outer face of the fruit base, corresponding to Position 2 or 3. Unseparated unripe zones were excised from longitudinal sections of the zone using a new, sharp, one-sided blade. The unseparated zone was not easily seen unless it had become translucent (compare Fig. 3.6A with 3.6B, page 82A) and it was impossible to dissect it completely free of the pedicel fibres below it and fruit mesocarp tissue above. The RA was dissected from the inner whorl of tepals (fruit shed 1+2) or from the fruit base (unseparated fruit, 1+3 or 1+4/5 shed fruit) with a single-edge dissecting needle. The inner tepal base 1 and outer tepal base 2 were collected separately; tepal base 1 has some attachment to the fruit, whereas tepal base 2 represents attachment to the pedicel only. In fruit which had separated 1+2, 1+3 or 1+2/3, the base of the inner tepal 1 corresponded to Positions 2 and 3. In fruit which had separated 1+4/5 no separation had occurred at 2/3 and the base of tepal whorl 1 corresponded to Position 4/5; the base of tepal whorl 2 corresponded to Position 5 only (Fig. 2.1). Samples of tepal base 1 from **separated fruit** were prepared by removing (with scissors) the tepal tissue above the position of the RA (Fig. 2.4B & C). Tepal base 2 was prepared in a similar way; the tissue at the base of the tepal was removed and collected (Fig. 2.4D). The tepals from the inner whorl 1 were easily distinguished from those of the outer whorl 2 because they differed in shape at their base. Tepal base 1 from **unseparated fruit** was prepared by removing (with scissors) the tepal tissue above the position of the RA and below the area where separation would occur, pedicel fibres were trimmed off (Fig. 2.4A). All the tissues were stored at -20°C.

2.4 HOMOGENIZATION AND EXTRACTION OF FRUIT TISSUE

Ammonium sulphate protein preparations of fruit tissues were prepared in the following way: Tissue (7-10g) was ground to a fine powder with liquid nitrogen. It was then homogenized with a Polytron MSE ATO Mix on Speed 1, 4 x 5sec over 1.5min in 20mM NaOAc, 2mM EDTA pH 4.5, 1:5 dilution (1g tissue + 4mls buffer). The homogenate was poured into glass Corex tubes (30ml) centrifuged at 9,700g max (Sorvall

Centrifuge SS-34 rotor) at 4°C for 20min. The pellet was washed once with 20mM NaOAc pH 4.5, centrifuged and the first and second supernatants combined. The combined supernatant contained soluble enzymes. The pellet was then resuspended in 500mM NaOAc/1M NaCl/2mM EDTA and stirred on ice for 2h. This was centrifuged as for the "soluble" enzyme preparation and the pellet also washed once and the supernatants combined. This supernatant contained cell wall desorbed enzymes. $(NH_4)_2SO_4$ protein precipitates were made from the "soluble" (20-40ml) and "cell wall" (50-60ml) supernatants. One cut only of 80% $(NH_4)_2SO_4$ was prepared for convenience. The precipitate after centrifugation was re-suspended in a minimum volume of 10mM NaOAc pH 4.5 and dialysed (2 x 1.5h) in 10 litres 10mM NaOAc in deionised water at 4°C in the cold room. After dialysis aliquots of the extracts were dispensed into Eppendorf tubes and frozen at -20°C. The separated zone extract after dialysis contained a thick gelatinous substance which appeared to have "precipitated out" in the cold. It was assumed to be pectins, and the separated zone was the only tissue to show this.

Enzyme analyses of these preparations indicated that there was some cellulase, polygalacturonase (PG), ß-galactosidase and ß-1,3-glucanhydrolase activity. However, this method of enzyme extraction was lengthy, especially when preparing several different tissue samples, and there may have been some loss of enzyme activity. Knegt *et al* (1988) reported that for purification of tomato polygalacturonase saturation with 80% ammonium sulphate resulted in appreciable losses in activity (up to one half). Therefore, the following procedure was used and by this method the time to prepare enzyme extracts was reduced to about 1h.

2.4.1 Preparation of Extracts for Enzyme Analyses

The information on the preparation of extracts from plants by Gegenheimer (1990) and Loomis (1974) was adapted for the extraction of oil palm tissue as described in the following protocol.

Unseparated zone sections, mesocarp, tepal bases, periphery and rudimentary androecium are the tissues which could be all ground to a fine powder in liquid nitrogen with a mortar and pestle. The mortar was then slowly returned to a temperature just above freezing and was placed on ice. The cells scraped off the fruit zone (FZ) and pedicel zone (PZ), that is, separated zone tissue, was not suited to grinding with liquid

nitrogen. It formed hard concrete-like balls which were extremely difficult to crush with the pestle and attempts to do so resulted in loss of precious tissue, so for the FZ/PZ tissue extraction, the mortar was pre-chilled at -20°C and then placed on the ice.

The extraction buffer was 250mM acetate buffer pH 5.0 containing 10mM DTT, 25mM $MgSO_4$ and 1M NaCl. Magnesium sulphate was included in the extraction buffer because it had been suggested to increase precipitation of pectins (Dr A.K. Kanellis, personal communication; Kanellis & Kalaitzis, 1992) and had no inhibitory effect upon the enzymes being analysed. Acid washed sand (0.5g g Fw^{-1} tissue, to improve cell disruption), insoluble PVP (0.05g g Fw^{-1} tissue, to adsorb phenolic compounds), and protease inhibitors were also added. The soluble protease inhibitors leupeptin and APMSF were used in preference to PMSF which is not very soluble in aqueous solution. Leupeptin inhibits serine and cysteine proteases and APMSF inhibits serine proteases. For each ml of total homogenate 1μg leupeptin and 30μg APMSF (prepared in deionised water) were added after grinding with liquid nitrogen at the time of the first buffer extraction.

Initially, the tissue was extracted twice with buffer and after the first extraction, the tissue was stirred with fresh buffer for 2h (at 4°C). However, higher activities were obtained when the tissue was extracted 3 times with the extraction buffer in succession, and by this method the time to prepare enzyme extracts was reduced to 30-60min. Separated zone (FZ/PZ combined; FZ or PZ alone) and unseparated zone were extracted 3x1.5ml. Mesocarp and tepal bases were extracted 1x2ml and 2x1.5ml. After each extraction the homogenate was centrifuged 2000g max for 5min (MSE Benchtop). The supernatant of the homogenate was kept on ice and the pellet was re-extracted with new buffer. The supernatants were pooled before a final centrifugation in the Beckman L60 Ultracentrifuge at 278,000g max at 4°C for 35min.

2.5 DESALTING AND CONCENTRATION OF ENZYME EXTRACTS

2.5.1 Desalting Enzyme Extracts by Gel Filtration

The enzyme extract after ultracentrifugation still contained 1M NaCl which made it not only unsuitable for electrophoresis, but also even 10μl of crude extract gave a very high background (an absorbance of 0.6-1.2 at 276nm) in the reducing sugar assay. This

was probably due to the presence of sugar monomers and oligomers which reacted with the cyanoacetamide (Fig. 2.5, Section 2.6.2).

To overcome these difficulties, Econo-Pac 10DG columns (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts.) were used in conjunction with a peristaltic pump (LKB 2120 Variopex II) and Frac 100 fraction collector (Pharmacia, St Albans, Herts). These pre-filled columns are packed with BioGel P-6DG (desalting gel) which excludes solutes greater than 6000 daltons, allowing them to elute in the void volume. The column was calibrated with blue dextran/dichromate. Columns were equilibrated and eluted with 50mM NaOAc pH 5.0. The supernatant from ultracentrifugation (3ml) was applied to the top of the column, 5x840μl (total 4.2ml) fractions containing >6000 MWCO (corresponding to the blue dextran fractions) were collected in 1.7ml Eppendorfs. It took 10mins to obtain the protein fractions. They were then pooled and this resulted in a 1.3 dilution of the original sample. The desalted sample was either immediately assayed or frozen at -20°C.

Activity of PG and ß-1,3-glucanhydrolase (laminarinase) was checked before and after gel filtration. There was no loss of activity after dilution of the extract was taken into consideration. However, the cellulase (ß-1,4-glucanhydrolase) activity in extracts was lost after gel filtration. It was thought that the enzyme may have been denatured by, or adsorbed to, the Bio-Gel P-6DG polyethylene resin. Moore & Bennett (1994) also used Bio-Gel P-6DG resin to desalt their extracts but they pre-treated it with 0.025% cytochrome c to prevent non-specific losses of PG isoenzymes to the matrix. For cellulase, therefore, active preparations were obtained by extraction of 1g of tissue in a 3ml dilution (2 x 1.5ml) and centrifuged in a Beckman Microfuge (5min) at 9,000g max. The supernatant was used directly in viscosity assays.

2.5.2 Ultrafiltration of Enzyme Extracts

Enzyme extracts desalted as described in Section 2.5.1 were too dilute for visualisation of protein by electrophoresis. Concentration of the extracts was necessary. Initially, spun-cell filters (eg Centricon, Amicon) which use the gravitational force generated in a benchtop centrifuge were tested. However, these always became blocked and filtration was very slow. Stirred-cell ultrafilters are used with a gas cylinder regulated up to 50psi pressure which provides the driving force for filtration. Filtron 10ml stirred

up to 50psi pressure which provides the driving force for filtration. Filtron 10ml stirred cells (Omega cell) ultrafilters (Flowgen Instruments Ltd, Sittingbourne, Kent), were therefore used with 3K, 10K and 100K molecular weight cut-off (MWCO). The ultrafilter was placed on ice during concentration. A maximum of 30psi pressure was applied by means of compressed nitrogen (oxygen free) gas (BOC). Even with the stirred-cell ultrafilters, when extracts contained a lot of pectin, the speed of ultrafiltration was quite slow. For example, 5ml of FZ/PZ extract took between 1-2h in a 100K ultrafilter.

In order to decrease the time of ultrafiltration and to reduce the concentration of pectins the enzyme extracts were first passed through ultrafilters of 100K MWCO. The collected filtrate from this was then concentrated in either a 10K or 3K MWCO ultrafilter to a volume of about 500μl for electrophoresis; 4ml of filtrate with the concentration of pectin reduced took about 1h to concentrate to 500μl in 10K or 3K ultrafilters. The 3K and 10K ultrafilters were tested to ensure that they retained the polygalacturonase (PG) and ß-1,3-glucanhydrolase (GH) activity. It appeared that PG was retained by the 10K MWCO and that ß-1,3-GH was smaller in size and retained only by the 3K MWCO as shown in Table 2.3.

	ENZYME ACTIVITY OF FZ/PZ EXTRACT (nmol/h)			
MWCO of Ultrafilter	RETAINED		FILTRATE	
	PG	ß-1,3-GH	PG	ß-1,3-GH
10K	16.9	34.6	1.9	24.8
3K	21.1	49.5	0.4	4.2

Table 2.3: Determination of Ultrafiltration MWCO for Concentration of Polygalacturonase (PG) and ß-1,3-Glucanhydrolase (ß-1,3-GH) Enzymes

Occasionally, assays of the concentrated extract indicated that it was less active than expected from calculation after concentration. This may have been due to actual loss of enzyme activity or to enzymes being adsorbed to the pectin which was removed in the 100K filtration.

The 3K and 10K stirred ultrafilters were also used to desalt **and** concentrate fractions from the ion exchange chromotography (Section 2.9). After an initial concentration to about 1ml the sample was diluted to 10ml with acetate buffer and

concentrated again to reduce the NaCl concentration. This was repeated once more for samples in 1M NaCl. The speed of ultrafiltration of these fractions was 1ml min^{-1} for the 10K ultrafilter, and 1ml $5min^{-1}$ for the 3K ultrafilter.

2.6 ASSAYS USED FOR DETERMINATION OF ENZYME ACTIVITY

2.6.1 Assay of Enzyme Activity by Decrease in Viscosity

Substrates such as carboxymethyl cellulose form viscous solutions and the measurement of viscosity change is extremely sensitive. A decrease in viscosity due to enzyme hydrolysis (endo-cleavage) of substrate detects activity which is below the sensitivity of the cyanoacetamide assay for reducing groups.

The assay was incubated and measured in a water bath at 30°C since viscosity can vary dramatically with temperature. The viscosity of the assay solution was determined by recording the time the solution took to flow a fixed distance through a pre-calibrated 100μl disposable pipette (Vitrex, Camlab, Cambridge, UK). The enzyme extract and substrate was dispensed by means of an Eppendorf Multipette 4780 into a disposable round-bottomed tube (75x10mm, type RT-30, Sterilin Ltd) and these calibrated pipettes remained in the tube during the assay so that they were not subject to any cooling which might alter the viscosity measurement. A stop watch which measured in 1/100ths of a second was used to determine the flow rate.

Enzymes measured viscometrically were cellulase (ß-1,4-glucanhydrolase), polygalacturonase and mannanase. To assay endo-polygalacturonase activity, polygalacturonic acid was made to a 10% solution in acetate buffer pH 4.5. However, when 50μl of enzyme extract was added to 500μl of substrate, the viscosity reduced markedly and low viscosity solutions were more difficult to assay accurately. Locust bean gum has been used as a substrate to measure endo-mannanase activity in tomato fruit (Pressey, 1997). This substrate forms extremely viscous solutions and a stock solution was prepared at a concentration of 1%. A viscometric assay for ß-1,3-glucanhydrolase was attempted but the laminarin substrate did not form a viscous solution suitable for use.

- **Assay for Cellulase Activity**

Cellulase activity was examined initially in ammonium sulphate protein preparations (Section 2.4). These assays showed that there was some cellulase activity in separated zone and ripe mesocarp extracts. The mesocarp extract was more active than the separated zone at pH 4.5 but activity was low. For example, in 4h there was a 48% decrease in viscosity in mesocarp compared with an 18% decrease in separated zone.

The cellulase activity in extracts, prepared as described in Section 2.4.1, was either negligible or not detected. However, activity was detected before desalting on a Biogel column. The loss of activity after gel filtration on Biogel appears to be due to adsorption or denaturation by the Biogel resin, since cellulase activity in enzyme extracts desalted on Sephadex G25 (Pharmacia) was unaffected.

Enzyme extracts which contained the most cellulase activity were prepared in the following way: Tissue was extracted with the least amount of buffer possible (1g + 3ml, 2 extractions of 1.5ml each) and centrifugation in a Beckman Microfuge (9000g, 1.5min). The sodium chloride in the extraction buffer (1M) was ~ 100mM in the assay, and this was shown to have no effect on cellulase activity, so that desalting was not necessary. The protease inhibitors, leupeptin and APMSF were used in the extraction of tissue as described in Section 2.4.1. Enzyme extract (50μl) was added to 400μl of a 2% solution of carboxylmethyl cellulose in water with 50μl of 1M buffer at the appropriate pH (final concentration 100mM). The pH-dependent activity was determined for cellulase with glycine/HCl, acetate and phosphate buffers. A control contained 50μl of extraction buffer only in 400μl CMC at the appropriate pH (50μl of 1M buffer) and no enzyme extract. The viscosity of this tube was always obtained at the same time as enzyme extracts. This ensured that any change in viscosity of the test solutions was due only to enzyme activity, rather than fluctuations in the temperature of the air or water bath. The viscosity of the control tube usually remained constant. Occasionally, there was an increase or decrease in viscosity but this was not more than 5%. Viscosity readings were started after a 15min equilibration period in the water bath (30°C) and taken at regular intervals; for ripe mesocarp every 15-30min for 1-2h, and for separated zone every 30-60min for 2-5h. Tissue extracts which had very low activity (unseparated zone, unripe mesocarp and tepal base) were incubated for longer periods. Tubes were covered in parafilm overnight and a final viscosity reading taken after 20-24h.

2.6.2 Assay of Enzymes forming Reducing Groups

Polygalacturonase, ß-1,3-glucanhydrolase (laminarinase) and other enzymes which form reducing sugars or groups were assayed by the cyanoacetamide method of Gross (1982). Ultraviolet-absorbing products (276nm) are formed when 2-cyanoacetamide undergoes a Knoevenagel condensation and cyclization with reducing sugars, or the reducing ends of sugar polymers. The neocuproine assay for reducing sugars (Chaplin & Kennedy, 1986) was also tested but it did not appear to be as sensitive as the cyanoacetamide assay. Furthermore, linearity for the standards, galacturonic acid and glucose, was not observed with the neocuproine assay.

The substrates used in the cyanoacetamide assay were polygalacturonic acid or pectin (10mg ml^{-1}) for endo- and exo-polygalacturonase; laminarin (30mg ml^{-1}) for ß-1,3-glucanhydrolase or laminarinase; locust bean gum (10mg ml^{-1}) for endo-mannanase; carboxymethyl cellulose (20mg ml^{-1}) for ß-1,4-glucanhydrolase or cellulase; xylan (10mg ml^{-1}) for xylanase; and gum arabic (2mg ml^{-1}) for hemicellulose hydrolysis. All substrates were washed extensively with 80% ethanol and dried prior to use. The pH-dependent activity for polygalacturonase, laminarinase and cellulase was determined. Substrates were then prepared in 50mM buffer at the pH optimum for the enzyme. An Eppendorf Multipette 4780 was used as it dispenses liquids with positive pressure displacement and is especially suitable for viscous substrates such as carboxymethyl cellulose, locust bean gum and gum arabic.

The assay with cyanoacetamide was linear with glucose to 300nmol (r^2=0.9998); with galacturonic acid to 200nmol (r^2=0.9999); and with galactose/mannose 1:5 to 100nmol. The standard curves were reproducible as seen in Table 2.4.

GLUCOSE STANDARD (2mM) n = 19			**GALACTURONIC ACID STANDARD (0.5mM)** n = 25		
nmol	Absorbance	Std Dev.	nmol	Absorbance	Std Dev.
0	0.051	± 0.023	0	0.069	± 0.026
100	0.408	± 0.046	25	0.325	± 0.030
160	0.626	± 0.046	50	0.577	± 0.032
200	0.786	± 0.057	75	0.821	± 0.037
300	1.136	± 0.068	100	1.065	± 0.046
	r^2 = 0.99976			r^2 = 0.99988	

Table 2.4: **Standard Curves for the Estimation of ß-1,3-GH (Glucose) and PG (Galacturonic Acid) Activity by the Cyanoacetamide Reducing Sugar Assay (Gross, 1982)**

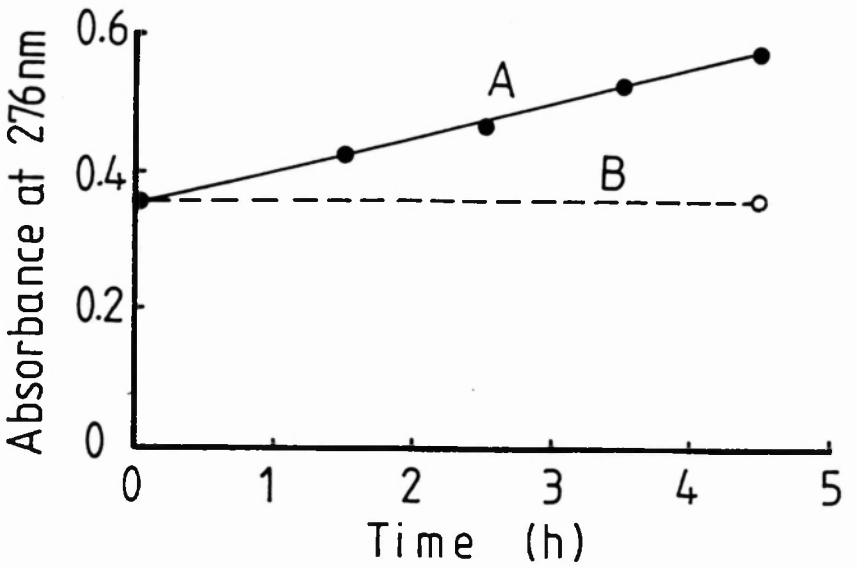


FIG. 2.5: ENDOGENOUS ACTIVITY IN ENZYME EXTRACTS OF SEPARATED ZONE

A. The increase in absorbance with time of an enzyme extract (separated zone) but with **no** substrate added. This activity was measured by the increase in reducing groups (cyanoacetamide assay, Gross, 1982) from 0-4.5h. (The activity of enzyme + endogenous substrate was measured in the control tubes (no added substrate) and then deducted from the total activity measured in the test (with added substrate)).

B. Enzyme extract denatured by heating in a boiling water bath for 15min. No endogenous activity remained after this treatment.

Activity in controls (no added substrate during the incubation period; substrate was added after the reaction was terminated) and extracts under test (tests) was calculated from the standard curve as nmol reducing groups formed per hour. The control value was deducted from the test value and the enzyme activity in nmol g Fw^{-1} min^{-1} was then calculated as described in Section 2.6.6. The small variation that occurs between individual standard curves did not alter the final result when the corresponding control value was deducted from the test value. This was checked by re-assaying extracts (which had been frozen) the following day or week. Also, when a new extract of the same sample was prepared, the assayed activity was essentially the same.

The following procedure for the cyanoacetamide assay of reducing groups is described for polygalacturonase. The other enzymes noted above were assayed in exactly the same way with their respective substrates and standards.

- **Assay for Polygalacturonase Activity**

The reaction mixture, in a total volume of 200μl (in Pyrex glass tubes 100 x 12mm), contained 10-50μl of desalted enzyme extract: separated zone FZ/PZ and fruit periphery, 10-20μl; unseparated cut-out zone, mesocarp, tepal bases and rudimentary androecium, 50μl. The enzyme solution volume was made up to 150μl with 50mM NaOAc pH 5.5 and 50μl of substrate, polygalacturonic acid (10mg ml^{-1} in 50mM NaOAc pH 5.5).

The enzyme extract contained enzyme as well as an uncertain amount of endogenous substrate such as water soluble pectin. Figure 2.5 shows that there was an increase in absorbance with time when enzyme extract and buffer alone was incubated for 0-4.5h. No increase in absorbance occurred with boiled enzyme extract. Therefore, assay controls (in duplicate) consisted of enzyme and buffer with no substrate. To start the assay, 50μl of substrate was added to the enzyme tests (in triplicate). The set of 5 tubes were mixed thoroughly, a glass marble was placed on the mouth of each tube to prevent evaporation and all were incubated at 30°C in a water bath (Tecam TE-7 Tempette) usually for 1 or 2 hours.

The assay was terminated by the addition of 1.25ml of cold cyanoacetamide/borate buffer solution. This was prepared immediately before use from 100ml of 100mM sodium borate/boric acid buffer pH 9.0 and 20ml of 1% cyanoacetamide. To the control tubes only, 50μl of substrate was then added.

The tubes were thoroughly mixed, and the tubes (with their marbles) were immersed in a boiling water bath. Galacturonic acid standards in duplicate containing 0-200nmol in a total volume of 200μl were placed with the controls and tests and boiled at the same time. After 15min the tubes were rapidly cooled to 20°C in a cold water bath. The absorbance of the samples was determined using quartz UV cuvettes (Spectrosil, 1.6ml) and a UV/Vis double-beam spectrophotometer (Cecil CE 4400) set at 276nm. The cyanoacetamide borate buffer solution in the reference cuvette was used to zero the machine.

2.6.3 Determination of exo-Glycosidase Activity

The exo-glycosidase activity was assayed by measuring the rate at which *p*-nitrophenol was released by hydrolysis of the synthetic α- or ß-*p*-nitrophenyl glycopyranoside substrate. This is a commonly used method for the assay of exo-glycosidases (Pharr *et al*, 1976). The *p*-nitrophenyl glycoside substrates used were:

p-nitrophenyl-α-D- and ß-D-glucopyranoside

p-nitrophenyl-α-D- and ß-D-galactopyranoside

p-nitrophenyl-α-D- and ß-D-mannopyranoside

p-nitrophenyl-α-L- and ß-L-arabinopyranoside

p-nitrophenyl-α-L-arabinofuranoside

p-nitrophenyl-α-D- and ß-D-xylopyranoside

p-nitrophenyl-ß-L-fucopyranoside

p-nitrophenyl-ß-D-cellobioside

The substrate was prepared at a concentration of 2mg ml^{-1} in buffer for enzyme assays. The pH-dependent activity of several glycosidases was determined for the separated zone (FZ/PZ) and mesocarp tissues using various buffers with pHs 2.4-8.1.

Initially, extracts prepared for the reducing sugar assays (PG and ß-1,3-GH) were used to estimate glycosidase activity (as described in Sections 2.4.1 and 2.5.1). However, it was found that higher activities were obtained when extracts were assayed directly after homogenization and a brief centrifugation (Beckman Benchtop Microfuge, 9000g, 1.5min). It was not necessary to desalt and remove small molecular weight substances by gel filtration because estimation of glycosidase activity with *p*-nitrophenyl-substrates is measured at 400nm. At this wavelength, the absorbance of the control (enzyme extract alone with substrate added only after the reaction was terminated) is low.

The reaction mixture in a total volume of 250μl (in disposable 75x10mm, 5ml round-bottomed tubes obtained from Sterilin Ltd, Type RT30) contained 30-50μl of desalted enzyme extract. The enzyme solution volume was made up to 100μl with buffer at the appropriate pH and 150μl of *p*-nitrophenyl glycoside (2mg ml^{-1}) was added to the tests (in triplicate) to start the reaction. Assay controls (in duplicate) contained enzyme and buffer only. The mouth of the tube was covered with a marble and the controls and tests were incubated at 30°C in a water bath for 15min to 4h depending upon the glycosidase assayed.

The reaction was terminated with 1.25ml of 0.2M sodium carbonate. This visualised the liberated *p*-nitrophenol which is best assayed spectrophotometrically under alkaline conditions at 400nm. For the controls 150μl of substrate solution was added after the reaction was terminated with sodium carbonate. The assay solution was transferred into a 1.6ml spectrophotometer cuvette (Sarstedt disposable 10x4x45mm cuvettes No. 67.742) and the absorbance of the controls and tests was measured in a UV/Vis spectrophotometer (Cecil CE 4400). Photolytic breakdown of phenylglycosides at alkaline pH has been reported (Pharr *et al*, 1976) and that 0.2M borate buffer pH 9.2 reduced this effect. However, the use of borate buffer was tested but it did not improve the assay; precipitation occurred in the borate terminated assays. In addition, these authors noted that the absorbance of assays terminated with sodium carbonate gradually increased. This was not observed in our experiments.

The activity of the exo-glycosidases was calculated by reference to a standard curve of *p*-nitrophenol 0-125nmol (Table 2.5) and activity in nmol g Fw^{-1} min^{-1} was calculated as described in Section 2.7.5.

p-NITROPHENOL STANDARD (0.5 mM) n = 10		
nmol	Absorbance	± Standard Deviation
0	0.060	± 0.004
25	0.339	± 0.040
50	0.660	± 0.045
75	0.978	± 0.061
100	1.296	± 0.078
125	1.590	± 0.119
	r^2 = 0.9996	

Table 2.5: Standard Curve for the Estimation of Glycosidase Activity

2.6.4 Determination of Pectin Methylesterase Activity

Enzyme extracts were prepared in deionised water containing 1M NaCl (1g tissue + 2x1.5ml 1M NaCl) with acid-washed sand, PVP and protease inhibitors (as described in Section 2.4.1). The extract was then assayed immediately by the continuous spectrophotometric method described by Hagerman & Austin (1986). The reaction mixture contained 10μl-50μl of enzyme extract, 1.5ml of 0.5% ethanol-washed citrus pectin in water containing 0.005% bromothymol blue indicator (pH 7.6-6.0, blue→yellow). The pH of the pectin solution was adjusted to 7.5 with 1N NaOH but the addition of the enzyme extract always lowered the pH and it was necessary to readjust it with ~1μl-2μl 50mM NaOH. The assay must always be started at the same pH to ensure reproducible colour changes and so the same initial absorbance value at 620nm of ~0.9-1.0 was used (pH 6.7-6.8). At this pH, PME activity was optimal; the gradual decrease in pH due to the formation of carboxylic acid caused the reaction rate to become non-linear. Therefore, PME was measured over a short period of time (1-2min) whilst the enzyme rate was linear.

The activity of PME was inhibited when the initial absorbance value was above 1.34 (pH $\geq$7.2). In addition, it was inhibited if the pectin substrate had not been washed extensively with 80% ethanol. This was first noticed in oil palm extracts and so a purified tomato pectin methylesterase (Sigma) also was tested. Complete inhibition of tomato PME occurred when the pectin had not been pre-washed in 80% ethanol. A 1% solution of the citrus pectin and the ethanol-washed pectin were compared for protein content:

1% pectin solution contains 6.3mg protein in 100ml.
1% EtOH-washed pectin solution contains 5.2mg protein in 100ml.

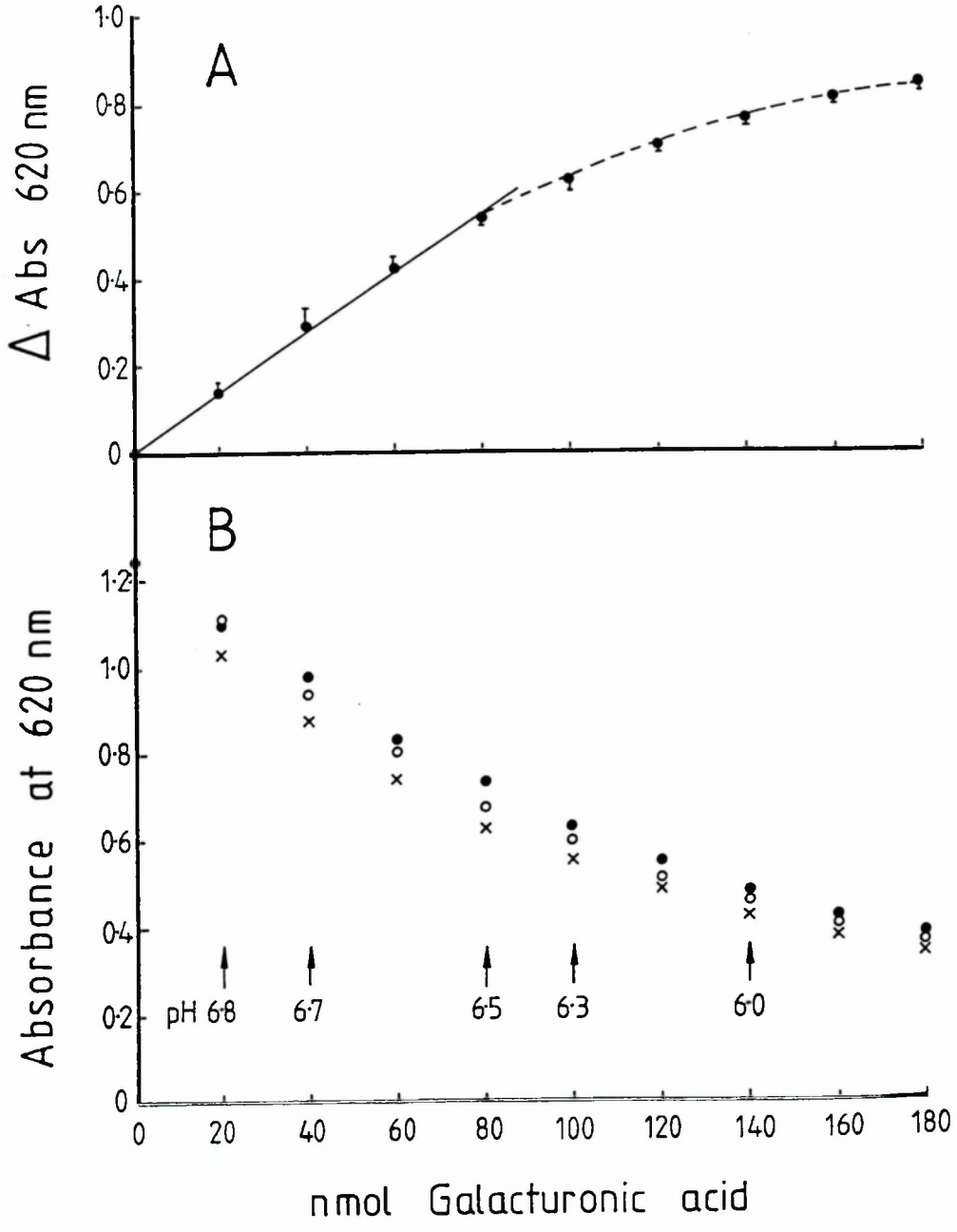


FIG. 2.6: CALIBRATION CURVE FOR THE SPECTROPHOTOMETRIC ASSAY OF PECTIN METHYLESTERASE ACTIVITY

A. The difference between A_{620} of a mixture of citrus pectin (0.5%) including bromothymol blue (0.005%) (1.5ml) and the A_{620} of a similar mixture containing 20-180nmol galacturonic acid (bar = s.d; n = 3).

B. The decrease in the A_{620} of a mixture of citrus pectin (0.5%) including bromothymol blue (0.005%) (1.5ml) with the addition of 20-80 nmol galacturonic acid. Three assays (shown on the graph as •,o,x) of the galacturonic acid standard were used to obtain the standard curve values in Table 2.6. The pH change at decreasing absorbance values is shown.

A glycoprotein inhibitor of pectin methylesterase in kiwifruit has been reported (Balestrieri *et al*, 1990) and something similar appears to be present in the pectin substrate (*Citrus*, Sigma). Whilst the 80% ethanol removed only some of the protein, it also probably denatured an inhibitor of PME.

The activity of PME was calculated by reference to a standard curve of galacturonic acid (0-80nmol) as shown in Table 2.6 and Fig. 2.6A (the standard curve was the same with HCl, a strong acid). The absorbance change at 620nm of bromothymol blue was monitored only in the linear region of the change in colour of the indicator (from blue-green to green-blue) (see Fig. 2.6B).

GALACTURONIC ACID (2 mM) n = 3		
nmol	Δ Absorbance	± Standard Deviation
0	0.000	± 0.00
20	0.139	± 0.02
40	0.284	± 0.03
60	0.423	± 0.03
80	0.539	± 0.03
	r^2 = 0.9985	
100	0.624	± 0.02
120	0.702	± 0.01
140	0.765	± 0.01
160	0.818	± 0.01
180	0.855	± 0.01

Table 2.6: Standard Curve for the Estimation of Pectin Methylesterase Activity

The assay solution was measured in a 1.6ml spectrophotometer cuvette (Sarstedt disposable 10x4x45mm cuvettes No. 67.742) and the continuous decrease in absorbance of the test solution was measured at 620nm in a UV/Vis double beam spectrophotometer (Cecil CE 4400) connected to a Servorgor 120 recorder (Servoscript (Lab Recorders) Ltd, Surrey).

To detect the PME activity in fractions eluted from ion exchange column chromatography, a 1% solution of ethanol-washed citrus pectin was prepared and 4ml of Universal Indicator was added to the 100ml. The pH was adjusted to pH 7.0 with 1N NaOH; 1.25ml of this solution was added to 150μl of fraction and the pH adjusted with

50mM NaOH if necessary. This method readily showed the presence or absence of PME activity.

2.6.5 Assay for Protease Activity

The activity of proteases in enzyme extracts can result in loss of enzyme activity. The protease activity in mesocarp and separated zone (FZ/PZ) extracts was examined by using the assay of Church *et al* (1985). This assay uses *o*-phthaladehyde and 2-mercaptoethanol which react with amino groups released during proteolysis. The reaction is specific for primary amines in amino acids, peptides and proteins, and forms a 1,2 disubstituted isoindole which absorbs strongly at 340nm. It is also quick and convenient and approaches the sensitivity of ninhydrin and other procedures.

The *o*-phthaladehyde (OPA) solution was prepared as described by Church *et al* (1985). It contained 50mM sodium borate, 0.1% (w/v) sodium dodecyl sulphate, 80mg of OPA (dissolved in 2ml methanol) and 200μl of 2-mercaptoethanol. Enzyme extracts of separated zone and mesocarp tissue were prepared as described in Section 2.4.1 but without the inclusion of the protease inhibitors APMSF and leupeptin. Enzyme extract, 10μl was incubated with 50μl of bovine serum albumin (2mg ml^{-1}) for a period of 0-5h. The reaction was terminated with 1.25ml of OPA solution. The enzyme and OPA solution was transferred to a 1.6ml quartz cuvette and the absorbance determined at 340nm in a UV/Vis double beam spectrophotometer (Cecil CE4400). OPA solution in the reference cuvette was used to zero the machine. No protease activity was detected in total extracts of either tissue.

The protease of *Streptomyces griseus* Type XIV was used as a positive control. It was prepared in 50mM acetate buffer pH 5.8 at a concentration of 1mg $2ml^{-1}$ and 10μl of protease enzyme was incubated with 50μl of BSA substrate. With these conditions, the protease activity measured by the OPA method was linear for 10min.

In addition, the effect of the presence or absence of protease inhibitors in assays for PG activity (cyanoacetamide assay for reducing groups) was examined. Separated zone extracts were assayed with and without leupeptin (20μl of 100μg ml^{-1}) or APMSF (20μl of 1mg ml^{-1}). No substantial difference in PG activity was observed in the assays with or without the protease inhibitors but for assurance the inhibitors were always included.

2.6.6 Calculation of Enzyme Activity in Extracts

- **Calculation of Activity in nmol from Standard Curves**

Mean absorbance values were calculated for the duplicate or triplicate controls, tests or standards. The mean absorbance values for known concentrations of galacturonic acid, glucose, *p*-nitrophenol, or protein (two-variable data pairs) were entered into a TI-36X calculator (Texas Instruments) and a regression analysis (linear equation, $y=a+bx$) used to predict the values in nanomoles for the controls and tests. The co-efficient of determination (r^2) for standard curves was not less than 0.999.

Enzyme activity in nmol calculated from standard curves could then be expressed as either per μg of protein or per gram fresh weight of tissue.

- **Protein Estimation in Enzyme Extracts**

When Coomassie Brilliant Blue G250 is bound by protein at an acidic pH, the wavelength at which it maximally absorbs light shifts from 465nm to 595nm. It is possible to use this change to assay protein concentrations (Bradford, 1976). This assay is sensitive enough to determine the protein concentrations of a sample containing as little as 1μg of protein. Bio-Rad produce a protein assay kit based upon this method. This was used because of its sensitivity and convenience according to the assay kit instructions, as follows:

> A quantity of the desalted enzyme extract: 10-20μl separated zone, 20-50μl mesocarp and unseparated zone, 100-250μl tepal bases; containing 2-10μg of protein was made to 250μl in disposable round bottomed tubes (75x10mm, 5ml Sterilin Ltd, type RT30) with 50mM NaOAc pH 5.0. The assay for protein in enzyme extracts was in duplicate. Then 1.25ml of the dye solution in buffer (0.95ml buffer and 0.3ml of Bio-Rad dye reagent) was added to give a total volume of 1.5ml (a final ratio of dye to enzyme extract and buffer solution of 1:5) and the tubes were thoroughly mixed. The assay was developed for between 5mins and 30mins. Each sample was transferred into a 1.6ml disposable spectrophotometer cuvettes (Sarstedt 10x4x45mm cuvettes, No. 67.742). The absorbance of the assay solution at 595nm relative to the absorbance of the buffer control in the reference cuvette was measured in a UV/Vis double beam spectrophotometer (Cecil CE 4400). The quantity of protein in the sample was

determined by reference to a standard curve of bovine albumin (Bio-Rad Standard II) which, in this assay, was linear from 0-12μg (r^2=0.9994). Protein standards were assayed in triplicate.

PROTEIN : BOVINE ALBUMIN STANDARD 14.2μg in 1ml n = 15		
μg	Absorbance	± Standard Deviation
0.0	0.534	± 0.024
2.84	0.660	± 0.033
5.68	0.793	± 0.040
8.52	0.906	± 0.039
11.36	1.033	± 0.044
	r^2 = 0.9994	

Table 2.7: **Standard Curve for the Estimation of Protein by the Coomassie Blue Dye-Binding Assay (Bradford, 1976).**

The rudimentary androecium and tepal base tissue contained barely detectable levels of protein. With some extracts of rudimentary androecium and tepal bases the assay required 500μl-1.2ml of enzyme extract in order to detect any protein. In separated zone FZ/PZ, unseparated zone and mesocarp extracts, protein values were obtained without difficulty. Table 2.8 shows the protein values obtained for these tissues:

Fruit tissue	μg protein ± standard deviation in μl extract	Extraction Dilution of tissue
Rudimentary androecium/tepal base 1 (n=23)	3.08 ± 3.16 in 500 μl	1g in 6 ml
Unseparated zone (n=5)	8.53 ± 2.06 in 50 μl	1g in 11 ml
Separated FZ/PZ (n=18)	11.12 ± 3.14 in 50 μl	1g in 11 ml
Mesocarp (n=8)	4.83 ± 0.73 in 50 μl	1g in 11 ml

Table 2.8: **Estimation of Protein in Fruit Tissues by the Coomassie Blue Dye-Binding Assay (with reference to a Standard Curve of a BSA Protein Solution, Table 2.8).**

These values indicate that the zone and mesocarp tissues appear to contain about 30-70 times more protein than the rudimentary androecium/tepal bases. However, the assayed enzyme activity of polygalacturonase or laminarinase (ß-1,3-GH) calculated on a protein basis did not appear to correlate with that observed when electrophoretic gels were stained for activity. For example, in an extraction of tepal bases for ripe normal separated fruit 1+4/5 and ripe mutant separated fruit 1+3 (the same weight of tissue was used for

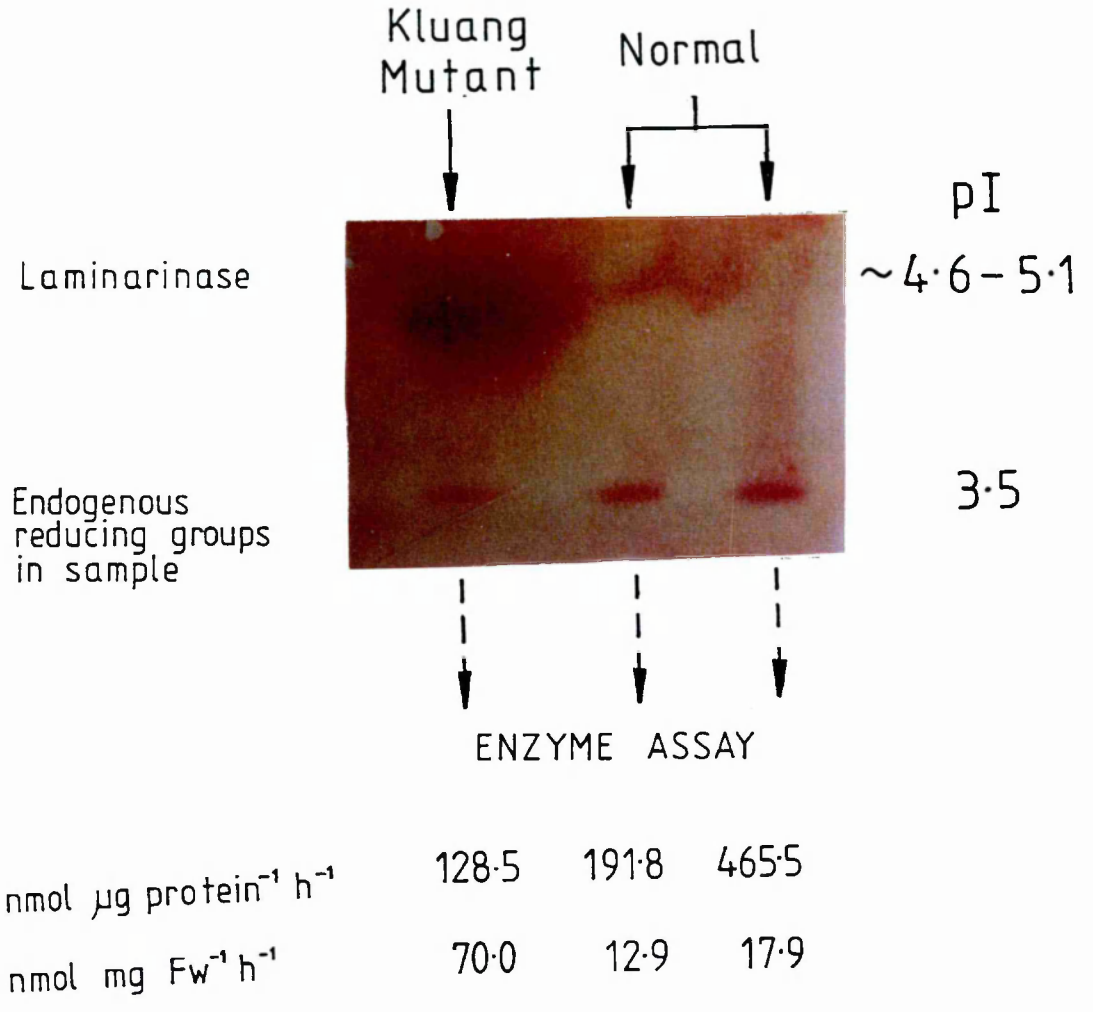


FIG. 2.7: LAMINARINASE ACTIVITY IN ENZYME EXTRACTS OF TEPAL BASE 1

The activity of the total extract was assayed by the cyanoacetamide method (Gross, 1982) and then calculated as either nmol reducing groups μg protein^{-1} h^{-1} or nmol reducing groups g Fw^{-1} h^{-1}. These activities were compared with the activity visualised on an IEF gel by staining with TTC for reducing sugars.

NB: The "band" at pI 3.5 is not due to enzyme activity because when incubated with an overlay moistened only with water (no substrate), staining (with TTC) is still obtained. It is possibly due to uronic acids from the hydrolysis of pectin in the enzyme extract (low molecular weight, negatively-charged reducing groups which migrate to the cathode).

each extraction) the activity was assayed for laminarinase (ß-1,3-GH) and then calculated on a per μg protein or per mg fresh weight basis. The results are shown in Table 2.9.

Enzyme Extract of Tepal Base 1	nmol μg protein^{-1} h^{-1}	nmol mg Fw^{-1} h^{-1}
Ripe Normal (separated 1+4/5)	465.5	17.9
Ripe Normal (separated 1+4/5)	191.8	12.9
Ripe Kluang Mutant (separated 1+3)	128.5	70.0

Table 2.9: Activity in Enzyme Extracts of Tepal Base 1 Calculated Either per μg of Protein in the Extract or per mg Fresh Weight of Tissue

These same extracts were fractionated by electrophoresis on an IEF gel (30μl) and the activity was detected by triphenyltetrazolium chloride (TTC) which reacts with and stains reducing sugars red (Section 2.7.1). The enzyme activity corresponded to that calculated per mg fresh weight of tissue as shown in Fig. 2.7 but the activity calculated per μg of protein was the opposite to that visualised on the gel. Several samples were checked in this way: from the enzyme activity visualised on an electrophoretic gel, compared with assayed enzyme activity calculated either per μg protein or per mg fresh weight of tissue, it was concluded that activity on a per mg fresh weight basis would give a more accurate estimation of enzyme activities of the different fruit tissues, and hence permit valid comparisons to be made between tissues.

Factors which might contribute to inaccuracies in estimated protein values include: Phenolic compounds are known to be present in zone and mesocarp tissue (Henderson & Osborne, 1990). The Hoepfner-Vorsatz test for polyphenols (Reeve, 1951) also showed staining at Positions 2, 3, 4 and 5, that is, below the rudimentary androecium and at the base of the tepals. These can contribute to an increased "protein" value, for example 50μl (1mg ml^{-1}) of 4-methylcatechol gives a "protein" value of 0.5μg. There is significant protein-protein variation with the Coomassie dye-binding protein assay. The major proteins of tepal base extracts may have had a lower absorbance than those in zone extracts. Therefore, protein estimation in total tissue extracts was **not** used as the basis for calculations of enzyme activity.

- **Estimation of activity on a fresh weight basis**

The dilution factor involved in homogenization and extraction of the tissue was calculated. For example, 500mg (equivalent to 500μl) of separated zone FZ/PZ is

extracted 3 times with 1.5ml of buffer and this is a dilution of 1g in 10ml. Another dilution occurred during desalting of the enzyme extract by gel filtration when 3ml was diluted to 4.2ml, that is, a dilution of 1.4. For example:

If the activity is 32.14nmol h^{-1} in 20μl of desalted extract, then

32.14 x 1.4 = 45.00nmol h^{-1} in 20μl original extract.

If original extract is a 1g + 9ml dilution ~10ml, then

20μl is 1/500th of the 10ml, therefore

Activity in 1g = 45.00 x 500nmol h^{-1}

$$\text{Activity in nmol g } Fw^{-1} \text{ min}^{-1} = \frac{22,500}{60} = 375.0$$

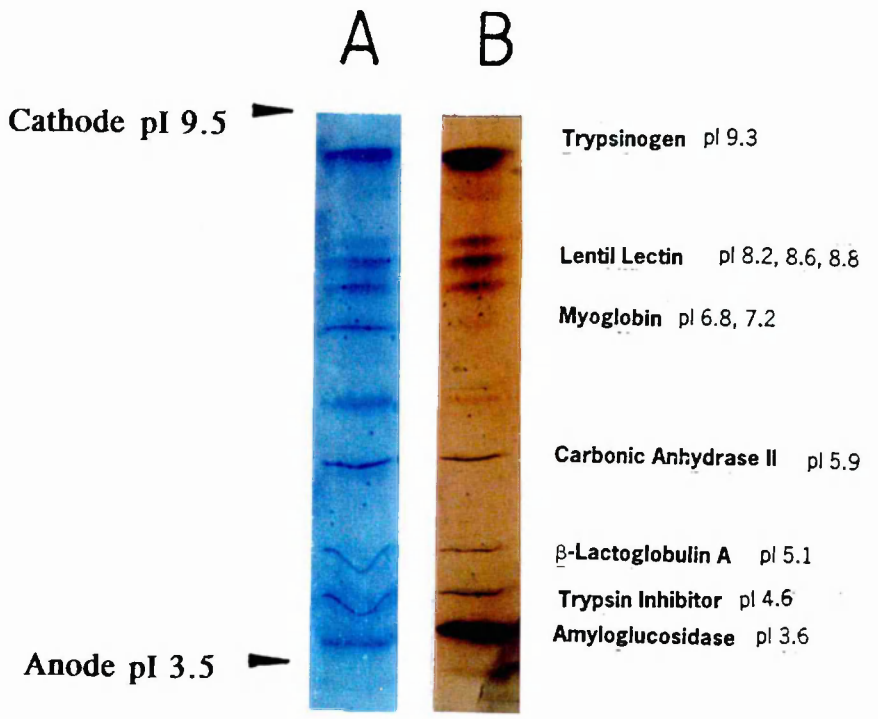
As a further check, the number of mg of tissue contained in the 20μl enzyme extract could be calculated. Although the results were similar to those calculated using the dilution factors, there was a greater chance of inaccuracy because the amount of buffer retained by the pellets of the different tissues can vary.

2.7 ELECTROPHORESIS

The majority of gels presented in this thesis were run on an Ultrophor Electrofocusing Unit (LKB 2217) with an Electrofocusing Constant Power Supply (LKB 2197). The Ultrophor unit was connected to a MultiTemp II thermostatic circulator (LKB) with the temperature set at 5°C and pre-cooled for 1h before electrophoresis.

Pre-cast gels on a plastic support film were used. They were isoelectric focusing Ampholine PAGplates and native CleanGels (Pharmacia). Gels were placed on the cooling plate with about 500μl of petroleum ether insulating fluid to ensure complete contact between the cooling plate and plastic backing of the gel with no air bubbles.

The isoelectric focusing gels (5% polyacrylamide Ampholine PAGplate pI 3.5-9.5, 1mm thick) could be cut into the desired width and power settings were adjusted according to the size of the gel. A full gel (24cm wide) was run at 10W power, with a starting voltage of ~300V. Smaller gels (7-14cm wide) were run at 5-7.5W power with starting voltage of ~200V. The voltage at the end of the gel run never exceeded 1500V. It was possible to fractionate 20 samples on a full gel, and each sample was loaded onto a 1cm applicator strip (Pharmacia). At times 20-40μl of sample was loaded onto 2 sample



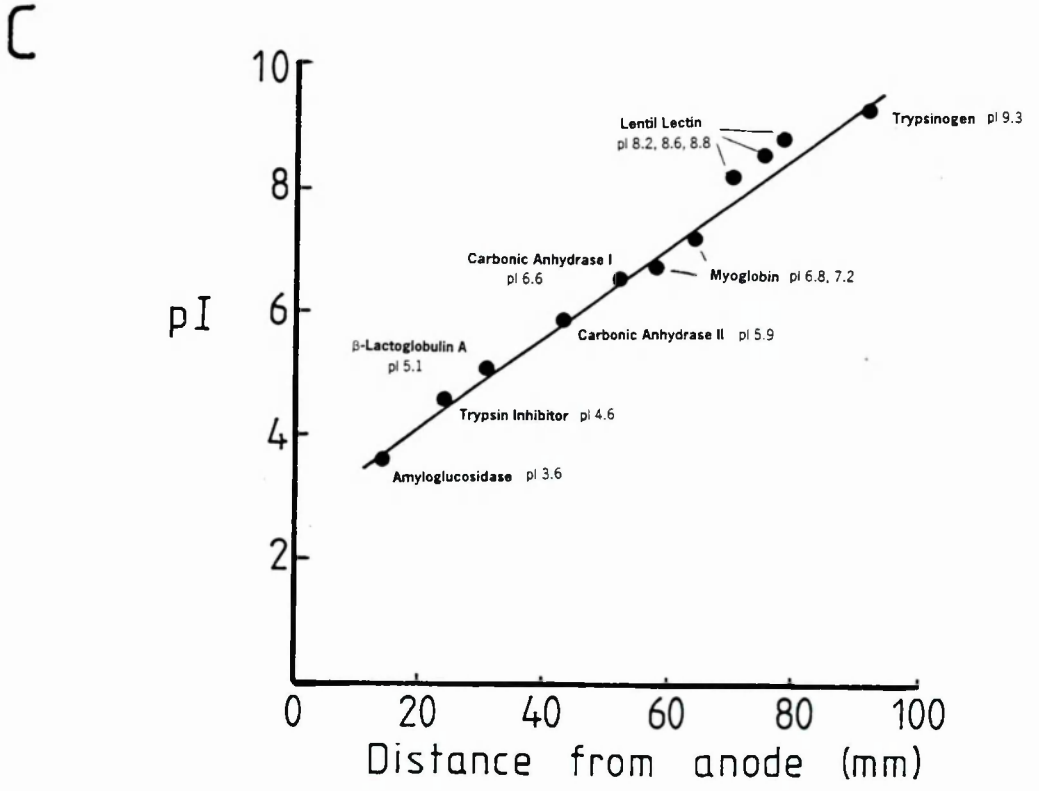


Fig. 2.8 CALIBRATION CURVE OF IEF STANDARD PROTEIN MARKERS PI 3.6-9.3

A. An IEF standard protein mix (20μl, Sigma) fractionated on an IEF gel pI 3.5-9.5 amd stained with Coomassie blue (R250).

B. As for (A) above but stained with silver (Protostain, Flowgen).

C. The standard IEF protein mix was fractionated on IEF gels (pI 3.5-9.5) and after staining with Coomassie blue (A) or silver (B) the distance from the anode (mm) of migration of each protein was measured.

application pieces placed on top of each other to increase the amount of protein applied. Each gel run took 1.5h: 30mins for pre-focusing, 30min with the sample on the applicator strip and a further 30mins after removal of the applicator strip from the gel. The end electrode buffer strips for the pI 3.5-9.5 gels were wetted with 1M NaOH for the cathode and 1M H_3PO_4 for the anode. A standard IEF protein mix (Sigma) with pIs between 3.6-9.3 was used to calibrate the pI of the PG isoenzymes (Chapter 6) and laminarinase (Chapter 7). Figure 2.8 shows the calibration curve of the standard proteins fractionated on pI 3.5-9.5 IEF gels (stained with Coomassie blue and silver).

The native gels (10% polyacrylamide CleanGel) were also used on the Ultrophor unit. These could also be cut to the desired width, and were used at half the size (12cm). These gels are supplied in a dry form and require rehydration for 1h prior to electrophoresis using the appropriate gel buffer supplied. For basic proteins and cathodic electrophoresis, the native buffer kit of pH 5.5 was used. For acidic proteins and anodic electrophoresis, the native buffer kit of pH 8.9 was used. Gel electrophoresis was carried out at a constant current of 20mA.

In addition, discontinuous gels with basic pH (Davis, 1964) or acidic pH (Reisfeld *et al*, 1962) were prepared for vertical electrophoresis in a mini Hoefer unit. However, the separations achieved were not an improvement on the above system.

2.7.1 Visualisation of Protein and Enzyme Activity on Electrophoretic Gels

- **Detection of Protein**

After electrophoresis, separated proteins in gels were fixed, according to the instructions for Ampholine PAGplates, in a solution of trichloroacetic acid (11.6%)/sulphosalicylic acid (3.4%) for 30mins. This also helped to wash out the ampholytes in the gel. Removal of the ampholytes was necessary because they are also stained by silver. The amounts of protein in extracts was low, even after concentration, and for satisfactory staining of a complex protein mixture with the Coomassie Brilliant Blue protein stain (PAGE Blue 83), it is necessary to load 50-100μg of total sample protein to the gel. Silver staining is up to 100 times more sensitive than the Coomassie protein stain and so less protein can be loaded on to the gel. A modified Coomassie stain (Neuhoff *et al*, 1988) was reported to have a sensitivity similar to the silver stain and it was tried, but the best results were obtained with silver stains. Those used included:

Silver Stain and Silver Stain Plus (Bio-Rad); Protostain (National Diagnostics, Flowgen); and Plus One silver staining kit for protein (Pharmacia).

Silver staining of the Ampholine PAGplate when ampholytes were removed appeared as black/brown bands on a clear background. Unfortunately, however, Pharmacia modified their gel casting procedure of the Ampholine PAGplate to enhance adhesion to the plastic backing sheet. These gels, when silver stained, had dark brown backgrounds. On investigation of the problem, the silver stain was shown to adhere to the plastic backing sheet. Removing the gel from the plastic backing during staining resulted in a clear background again but the gel was fragile and swelled after removal from its plastic backing sheet. The problem finally was resolved when the sulphosalicylic acid was omitted in the protein fixation step, and the protein in the gel was fixed in a solution of trichloroacetic acid alone (20%) for 2h.

- **Detection of Enzyme Activity**

For the activity staining of enzymes on IEF gels it was also necessary to wash out the ampholytes. These were removed by washing the gels 3 times (5min each time) in 50ml acetate buffer pH 5.0. The gels were gently rotated in the buffer on a rocking table (Luckham 4RT, Luckham Ltd, Sussex).

Since IEF gels are only 5% acrylamide, when they are stained for enzyme activity there is a problem of diffusion of the resulting low molecular weight products. To minimise diffusion, gels were not immersed in substrate solution directly but instead were overlaid with a single layer of filter paper pre-washed in deionised water and patted dry. The paper was then wetted with about 1-3ml of substrate solution: either PGA (2mg ml^{-1}), laminarin (30mg ml^{-1}) or *p*-nitrophenyl-ß-galactopyranoside (2mg ml^{-1}). The gel was placed in a sealed plastic box and incubated in a water bath at 30°C for 1-2h.

Reducing sugars were visualised by the method of Pan *et al* (1989) using triphenyltetrazolium chloride (TTC). TTC (300mg) was dissolved in 1M NaOH (200ml) prepared in deionised water (water which contains metal ions reacts with the TTC and a dark red precipitate forms on heating). The TTC/NaOH solution was warmed in a dish in a microwave oven (Menumaster Commercial 3100si, 1500 watts) for 20sec on 100% power. Then the gel was immersed in the warm solution and microwaved for 20sec on 100% power, 2 or 3 times until pink bands of activity appeared and the gel background

was not too dark. The gel was washed thoroughly in deionised water to remove the NaOH (if the NaOH is not removed the pink bands fade).

Activity staining of enzymes separated in the 10% native CleanGels was used for PG isoenzymes with pIs above 7.0. At a more basic pI (>7.0), these isoenzymes had greatly reduced activity, and on IEF gels they could not be visualised by activity staining with TTC. This did not reflect the activity of these isoenzymes known to be present from the cation exchange chromatography. Fractions from the ion exchange column (Section 2.9) were pooled and concentrated (Section 2.5.2). After electrophoresis the gel was equilibrated in 50mM acetate buffer pH 5.5. The gels were overlaid with filter paper wetted with PGA (2mg ml^{-1}), placed in a sealed plastic box to prevent drying out, and incubated at 30°C overnight. The activity was visualised by the method of Taylor *et al* (1993). The filter paper was removed and the gel rinsed with deionised water. The gel was then stained in a solution of 0.13% methylene blue in 10mM ammonium acetate pH 8.6 for 15mins, and destained in several changes of deionised water. Areas of activity were clear, whilst the rest of the gel remained blue.

For cellulase activity, carboxymethyl cellulose substrate was used by employing the same method as described above. To visualise activity, a solution of 1% Congo Red in acetate buffer was used and destained in 1M NaCl (Kanellis *et al*, 1991). However, this method did not prove to be successful for the oil palm cellulase although avocado cellulase could readily be demonstrated.

2.8 PREPARATION AND DEVELOPMENT OF WESTERN BLOTS

2.8.1 Buffers, Solutions and Agents used in Immunological Procedures

Phosphate buffered saline (PBS) was prepared by adding to 1 litre of deionised water: NaCl (8.0g), KH_2PO_4 (0.2g), anhydrous Na_2HPO_4 (1.15g), KCl (0.2g). PBS-Tween was made by addition of 0.5ml of Tween 20 per litre of PBS. Blocking buffer was prepared by adding to PBS: 1% bovine serum albumin (w/v), 1% chicken ovalbumin (w/v), 1% St Ivel's 5 Pints (w/v), 5% polyvinylpyrrolidone (w/v). The nitrocellulose sheet (0.45μm) was from Schleicher & Schuell (Dassel, West Germany).

Antibodies used were all prepared in blocking buffer. They were:

(i) Polyclonal antibodies to tomato fruit endo-polygalacturonase and pectin methylesterase obtained as a gift from Dr G. Tucker, University of Nottingham, Sutton Bonington. They were prepared at a 1/800 dilution. In addition, a further purified tomato fruit endo-polygalacturonase antibody preparation donated by Dr G. Tucker was used, and this was prepared at a concentration of 1/100.

ii) Polyclonal antibodies to the pI 9.5 bean abscission cellulase were provided by Dr R. Sexton, University of Stirling, and were prepared at a dilution of 1/800.

iii) Polyclonal antibodies to avocado and tomato cellulase from Dr Alan Bennett, University of California, Davis, prepared at dilutions of 1/5000 (avocado) and 1/500 (tomato).

2.8.2 Electrophoresis and Western Blotting

The Phast system (Pharmacia) requires protein samples containing at least 1μg of protein in a maximum of 4μl volume with a buffer concentration of less than 20mM. The samples were desalted on a Biogel column and concentrated by polyethylene glycol 6000. The flakes were packed around the sample in dialysis tubing (MWCO) 12-14,000 for about 30min. Once the protein samples had been prepared using Separation Method No.2 (isoelectric focusing between pI 3 and pI 9), they were fractionated as recommended by Pharmacia on Pharmacia IEF 3-9 Phast gels. Two gels were run at the same time and after electrophoresis, one gel was silver stained using the system staining method No. 1, and the other gel was used to produce a Western blot. The latter gel was chilled in a fridge at 8°C, and then it was eased gently onto a piece of nitrocellulose wetted with 10mM NaOAc buffer (pH 4.5) ensuring that there were no air pockets between the gel and the nitrocellulose. The gel was left on the nitrocellulose for 10min then carefully peeled off and developed as described in Section 2.8.3.

The Ultrophor/Multiphor System (Pharmacia) was used with isoelectric focusing (IEF) gels to separate proteins as described in Section 2.7. The IEF gel was a pre-cast Ampholine PAGplate which is a 1mm thick polyacrylamide gel cast on a plastic support film. The pI range used was 3.5-9.5. After electrophoresis, the gel was rinsed in deionised water and a piece of nitrocellulose which had been soaked in PBS-Tween was

placed on the surface of the gel. A piece of plastic and several layers of paper towelling were placed on top and then a constant gentle pressure applied for about 15min. The nitrocellulose was then carefully peeled off and developed as described in Section 2.8.3. Electroblotting as described in the Multiphor Electrophoresis System Users Manual, Sections 4J and 5K (Pharmacia), with the NovaBlot electrophoretic transfer kit was also used. The results were the same as for the manual method.

2.8.3 Development of the Western Blot

The Western blot was soaked in a solution of antibodies at a dilution specified for the enzyme as described in Section 2.8.1. The solution (~10ml) was placed together with the blot in a clip-seal polythene bag (15 x 10mm). After checking the bag for leakage, it was rotated on a rocking table at room temperature (this was done for all subsequent steps in the procedure) for 18h. The blot was rinsed thoroughly with PBS-Tween and soaked in blocking buffer for 3h and again rinsed in PBS-Tween. The blot was then soaked again in a second antibody (prepared in blocking buffer) for 3h.

(i) For the tomato PG, tomato PME, avocado and tomato cellulase the second antibody was goat anti-rabbit immunoconjugated peroxidase at a concentration of 1/5000.

(ii) For the pI 9.5 bean abscission cellulase the second antibody was also the goat anti-rabbit immunoconjugated peroxidase at a concentration of 1/5000.

After incubation with the second antibody, the blot was rinsed thoroughly with PBS-Tween and further soaked in PBS-Tween for 30min.

The areas of peroxidase activity were visualised by immersing the blot in 50mM Tris/HCl buffer (pH 7.4) containing 0.1% hydrogen peroxide (v/v) and 0.06% 3,3′-diaminobenzidine. The reaction was stopped by rinsing the blot in 50mM Tris/HCl buffer pH 7.4 and then transferring it to PBS containing 0.02% sodium azide (w/v).

2.9 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography was used in the separation of the polygalacturonase isoenzymes, laminarinase (ß-1,3-GH), ß-galactosidase and pectin methylesterase. The ion exchange resins used were: anionic, Q-Sepharose Fast Flow (Pharmacia); and cationic,

Express Ion C (Whatman). An Econo-column (30ml, Bio-Rad) was filled with 17ml of the resin. It was connected to a peristaltic pump (LKB 2120 Variopex II) and Frac-100 fraction collector (Pharmacia). Fractions were collected in 1.7ml Eppendorf tubes and 150μl of each fraction was assayed for polygalacturonase activity. The rest of the fraction was frozen at -20°C. Fractions with enzyme activity were then pooled and concentrated by ultrafiltration in a 3K stirred-cell as described in Section 2.5.2.

- **Anion Exchange**

  The Q-Sepharose resin was equilibrated in 20mM triethanolamine pH 7.7 and enzymes were eluted by changing the pH with 50mM histidine pH 5.0 and then by step-wise increases in the molarity of histidine to 100mM and 200mM. Fractions of 1.3ml were collected every 1.5min. The pectin in the extract was strongly adsorbed to the anion exchange resin and eluted with 500mM NaCl.

- **Cation Exchange**

  The Express-Ion C was equilibrated in 20mM NaOAc pH 5.5 and initially elution was with a step-wise gradient of NaCl: 25mM or 50mM, 100mM and 500mM. The column was washed with 1M NaCl and re-equilibrated with the 20mM acetate buffer. Fractions of 1.7ml were collected every 1.9min. The pectin in the extract was not adsorbed to the cation exchange resin and eluted in the void volume and initial buffer wash (20mM acetate).

2.10 MEASUREMENT OF ETHYLENE BY GAS CHROMATOGRAPHY

Ethylene analysis was by gas-solid chromatography on a Pye-Unicam series 104 gas chromatograph fitted with a flame ionization detector and hydrogen and air ignition gases. An alumina F1 column with nitrogen carrier gas was used for separation with analysis temperature and flow rates as described by Ward *et al* (1978). Whole spikelets of clone 926 were enclosed in 1.5l Kilner jars with a Suba-Seal inserted into the lid; and individual fruit of clone 926 were held in 28ml glass vials sealed with a Suba-Seal (No. 45, 20.5mm). Individual fruit of clone 271D and the non-abscinding mutant fruit were held in 60ml glass jars, also sealed with a Suba-Seal (28mm, No.62). For each determination, 1ml of the gas phase within each container was drawn through the Suba-Seal with a gas-tight hypodermic syringe. Results are expressed as nanolitres of ethylene produced per gram fresh weight per hour.

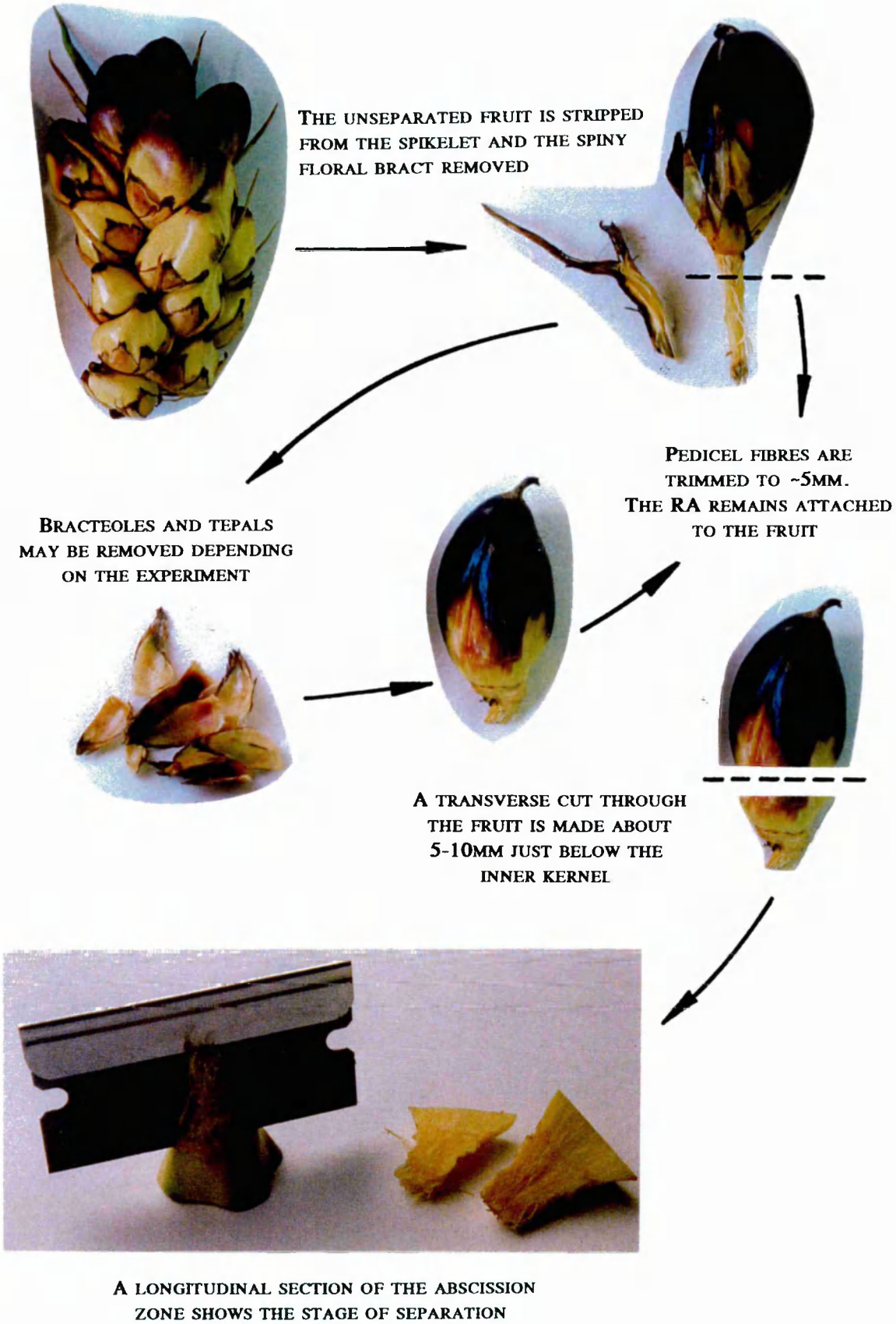


FIG. 2.9: PREPARATION OF AN UNSEPARATED FRUIT FOR THE MEASUREMENT OF ETHYLENE PRODUCTION AND THE SUBSEQUENT LONGITUDINAL SECTION TO DETERMINE THE STAGE OF ABSCISSION

2.10.1 Ethylene Production Studies

Ethylene production by shed or separating normal fruit was always high and was measured after a series of 30min incubations. Longer incubations (1h or more) were necessary to detect production in ripening or unripe normal fruit. Production was monitored from the time of arrival in the laboratory and thereafter at various intervals for the next 48h. During the aeration periods between measurements, containers were covered with Parafilm.

(a) Individual separated ripe fruit were those which either had shed during transit or which easily detached from the spikelet on arrival in the laboratory. These fruit were "naked" with no adhering tepals and traces only of the rudimentary androecium still attached to the fruit base.

(b) Individual ripe non-separated fruit with their attendant tepals were stripped from the spikelet and the fibres of the pedicel trimmed to 5mm below the abscission zone. The tepals were either completely removed to give a fruit with only the rudimentary androecium remaining attached, or the fruit was used with all tepals still intact. After ethylene measurement, a longitudinal section of the fruit zone was made to determine the stage of separation at the abscission zone. These manipulations are shown in Fig. 2.9.

2.11 HISTOCHEMICAL AND CYTOCHEMICAL PROCEDURES

2.11.1 Visualisation of Lignin by Phloroglucinol/HCl

Phloroglucinol in HCl (the Wiesner test) is generally considered indicative of coniferaldehyde end-groups in guaiacyl lignins, and stains a deep red colour (Lewis & Yamamoto, 1990). Lignin is found principally in sclerenchyma, in the tracheids and vessels of the xylem. In monocotyledons, the lignin is composed of equal amounts of coumaryl, coniferyl (which gives rise to the guaiacyl group) and sinapyl groups (Brett & Waldron, 1996).

Longitudinal sections through non-separated ripe zone were hand cut from fresh tissue, using a new, sharp, one-sided blade. The thickness of the sections was between 300-600μm. They were fixed in ethanol:glacial acetic acid (3:1) with several changes of fixative until the orange carotenoids were removed. The sections were then washed in

deionised water and each individual section was transferred to 500μl of a saturated solution of phloroglucinol in ethanol for 5min and then a drop of 35% HCl was added to it. The xylem vascular elements and fibres in the section then stained a deep red. Photo-micrographs were taken immediately using a Wild Heerbrug Makroskop 420 and Kodak 160 Tungsten positive film (slide film).

In order to calculate the relative area of the parenchyma and vascular/fibre elements, the slides obtained were enlarged by using a slide projector and projecting the image onto an A4 sheet of paper attached to a wall. The larger image of the sections showing the stained vascular/fibre elements of the fruit zone, pedicel below the zone and mesocarp above the zone were traced onto the paper. It was then possible to measure (with a ruler) the stained vascular/fibre elements relative to non-stained parenchyma tissue and a percentage was calculated.

2.11.2 Visualisation of Polygalacturonates in Pectin

The method used was as described by Varner & Taylor (1989). Calcium ion (Ca^{2+}) binds to uronic acids and the nickel ion (Ni^{2+}) will do the same. Cell walls which bind nickel ions can be visualised by addition of sodium sulphide and this forms a black nickel sulphide precipitate.

Longitudinal sections through non-separated ripe zone tissue were hand-cut from fresh tissue using a new, sharp, one-sided blade. The thickness of the sections was between 300-600μm. They were washed in 0.5% Nonidet P-40 (NP-40) for 30min. If then transferred to a 5-fold diluted solution of bleach, NaOCl (purchased from a local grocery) for 30min, the methyl esters of pectin are hydrolysed and the whole section then stains black. Therefore, in order to discriminate between methylated and unesterfieid pectin, the bleach stage was omitted.

Sections were transferred into a 0.05% solution of NP-40 for 30min and then directly into 10mM $NiCl_2$ for 30min. They were washed in deionised water to remove any unbound $NiCl_2$ and then placed in a solution of 0.1% solution of sodium sulphide. The unesterified pectin stains a dark brown to black colour within a few seconds.

2.11.3 Feulgen Staining and Microdensitometry

The Feulgen reaction is a widely used quantitative cytochemical procedure for DNA. It is based on the selective hydrolysis of purines by mild acid treatment which leaves aldehyde groups on the deoxyribose sugar moieties of the nucleotides exposed. The resulting 'apurinic acid' is then reacted with the leuco-basic fuchsin Schiff reagent which recolours (to purple) in the presence of aldehydes.

Cells were scraped from ripe separated fruit pedicel and fixed in 6 changes of Carnoy's fixative ethanol:glacial acetic acid:chloroform (6:1:3) over 18h at 20°C. They were hydrolysed with 5N HCl, 10min at 60°C or 15min at 20°C and then Feulgen stained for 1.5h (de Tomasi, 1936).

Measurements of nuclear DNA content were made with a Vickers M85 microdensitometer with the scanning light spot set at 565nm. Onion root tips were similarly Feulgen stained. The picograms of DNA per 2C onion cell is accepted to be 33.5pg (Bennett & Smith, 1976) and this was used as a standard to estimate the picograms DNA per cell in oil palm abscission zone cells.

2.12 URONIC ACID ESTIMATION

The method used to determine uronic acids is that of Blumenkrantz & Absoe-Hansen (1973). This method has been modified specifically to measure pectin in plant cell walls by Ahmed & Labavitch (1977) and more recently Filisetti-Cozzi & Carpita (1991). Both methods were tried; the latter method appeared to be more stable with time.

Freshly scraped cells of separated zone, the mesocarp tissue above and the fibres below the zone, were dried in an oven at 40°C for 24h. Each tissue was then ground to a fine powder with a mortar and pestle. The powder was used first in the ^{13}C NMR experiments (Section 3.5.3). After this the uronic acid content in the sample was determined. The powders were first extracted with solvent to remove carotenoids and non-polar compounds; chloroform-methanol and acetone extracts have been extensively used to prepare pectin rich cell walls (Koch & Nevins, 1989). The solvent did not remove the pectins, and re-grinding the tissues in the solvent produced a fine white powder suitable for acid hydrolysis. The dried ground tissue (~200mg) was extracted (3x) with $CHCl_3$:MeOH (1:1); it was then extracted with acetone (3x), and air dried.

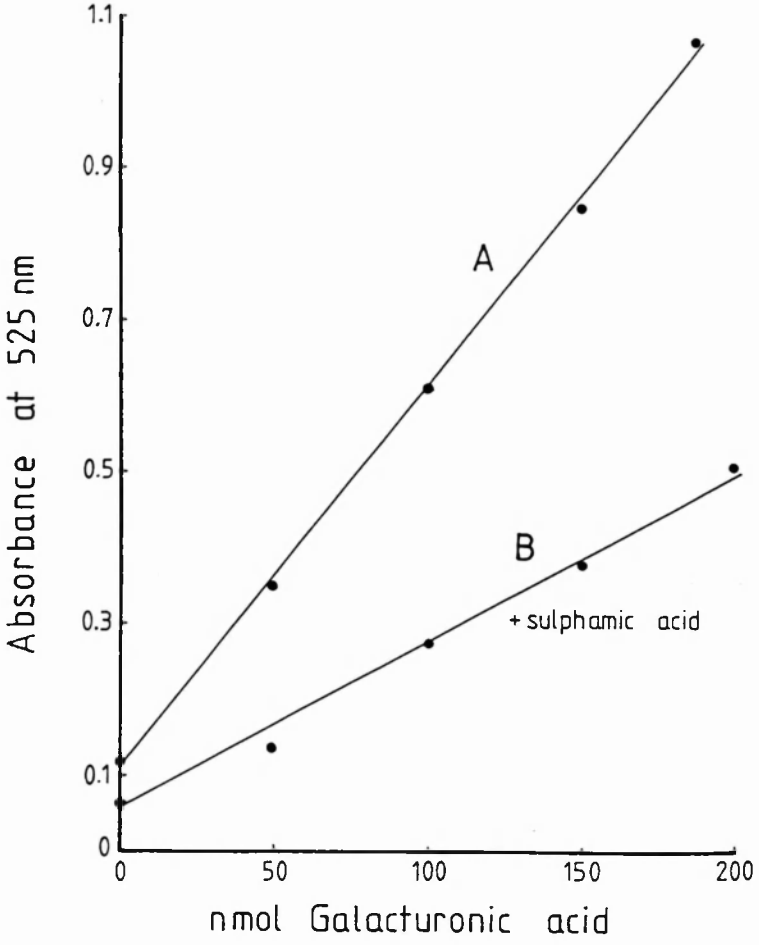


FIG. 2.10: CALIBRATION CURVE FOR THE SPECTROPHOTOMETRIC ASSAY OF URONIC ACID

A. The calibration curve of 0-200nmol galacturonic acid by the method of Ahmed & Labavitch (1977) as described in Section 2.12 ($r^2 = 0.9994$).

B. The calibration curve of 0-200nmol galacturonic acid by the method of Filisetti-Cozzi & Carpita (1991) which includes 40μl of 4M sulphamic acid/potassium sulphamate pH 1.6 ($r^2 = 0.9902$). The inclusion of sulphamic acid prevents the browning reactions that occur in the presence of neutral sugars.

A 5mg sample of the dried, solvent-extracted, ground tissue was weighed accurately. This was added slowly to 2ml of concentrated sulphuric acid in a 20ml beaker, stirred on ice. In order to prevent heating (with subsequent charring of the carbohydrate), deioinised water (0.5ml) also was added very slowly; after 5min another 0.5ml of deionised water was added dropwise. This was then transferred to a 10ml volumetric flask and with repeated washings of the beaker, the volume was made up to 10ml. For the separated zone the 5mg dissolved easily but for the mesocarp and fibres, it appeared that the lignified elements (~50% of the tissue) did not dissolve.

The tissue samples (0.4ml) and galacturonic acid standards (0.4ml; 0-200nmol in water, were dispensed into Pyrex glass tubes (125x15mm); then 2.4ml of concentrat H_2SO_4 containing 75mM sodium tetraborate was added and the solution stirred vigorou by vortex mixing. The tubes, capped with marbles to prevent evaporation, were pla in a boiling water bath for 20min. They were then cooled immediately in a cold w bath and, after cooling, 80μl of 0.15% (w/v) *m*-hydroxydiphenyl in 0.5% (w/v) N was added and stirred vigorously by vortex mixing. The pink colour develc completion in about 5-10min; the absorbance was read after 15min at 525nm an checked once more when all tubes had been read. Carbohydrates produce a sh chromogen with sulphuric acid/tetraborate at 100°C. Therefore, a blank sample co ing 80μl of 0.5% NaOH (without *m*-hydroxydiphenyl) was prepared for each te The amount of uronic acid equivalents was calculated by reference to the standard e of galacturonic acid (Fig. 2.10).

2.13 SOLID STATE ^{13}C CP-MAS NMR SPECTROSCOPY

Cross polarisation-magic angle spinning (CP-MAS) solid state ^{13}C spectra can be used to determine differences in the nature of polysaccharides *in s* the cell wall. Thus, the pectin of the abscission zone region could be analysed fu

2.13.1 Preparation of Samples

Standards of polygalacturonic acid and pectins with a k degree of methylesterification were as follows: (i) pectins (from lime, a gift frc rus Colloids Limited, Hereford) with a high degree of methylesterification (DM 70.8% (MW 546,000) and a low DM of 31.8% (MW 425,000); (ii) polygalactur icid potassium

salt (from orange, Sigma) with a DM of $<1.0\%$. The high and low methylesterified pectins from lime were acid-washed. Polygalacturonic acid potassium salt was washed with 80% ethanol and air-dried.

The mesocarp of normal unripe (61 daa), ripening (124 daa) and ripe (152 daa) fruit were compared. In the ripening fruit sample, the unseparated zone, the mesocarp above, the pedicel tissue below and the separated zone cells FZ/PZ also were compared. In addition, separated zone cells FZ/PZ of the Kluang mutant were analysed. Observations were directed to the resonances from the pectin present in the tissues. Since a considerable amount of water-soluble pectin was always extracted from the separated zone and mesocarp tissue when prepared for enzyme assays, the samples for ^{13}C NMR spectroscopy were oven dried at 40°C so that pectin present in the cell walls would not be removed.

For the comparison of unripe, ripening and ripe mesocarp, samples were taken from the mid-region of normal fruit (clone 271D). For the samples of the abscission zone and the tissues above and below it, normal ripening oil palm fruit (124 daa) were kept for 48h after arrival to allow the zones to become translucent. The unseparated zone was then excised; the mesocarp tissue directly above the zone and pedicel below the zone were also obtained. Some fruit were kept for a further 24h and after separation (at 72h), FZ and PZ cells were scraped from the zone. Samples of this slower separating zone of young ripening normal fruit were thought to be more comparable to the slow separation of harvested ripe Kluang mutant fruit (201 daa), from which FZ/PZ cells were collected and which had also separated after 72h. All the samples of tissues were oven-dried at 40°C for 24h and then, with a mortar and pestle, ground to a fine powder.

2.13.2 Solid State ^{13}C CP-MAS NMR Analyses

The spectroscopic analyses were carried out by Dr Stephen J. Heyes of the Inorganic Chemistry Department, Oxford University. Details of the experimental conditions are described in Appendix A.

2.14 LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

The preparation of oil palm sections for light and electron microscopy (photographs of which are presented in Sections 3.1.2 and 3.2.1) was carried out by Ms

Heather Davies of the Open University Electron Microscopy Unit, Milton Keynes. The tissues used were the mesocarp and zone of ripe separated (160 daa); ripe unseparated/separating (147 daa); and unripe unseparated (138 daa) fruit.

Details of the fixation and sectioning of oil palm tissues for light and electron microscopy is described in Appendix B.

2.15 EXTRACTION AND ESTIMATION OF MESOCARP CAROTENOIDS

Ikemefuna & Adamson (1984) have measured the carotenoid content in Nigerian oil palm mesocarp and the same procedures were used in our extraction and estimation of carotenoids in Malaysian oil palm mesocarp. A wavelength scan of the solvent-extracted carotenoids confirmed that α/ß carotene was the major absorption peak. After this standardisation, Ms Anne Norville carried out the estimations in normal (unripe, ripening and ripe); and the Kluang mutant (ripe) fruit. The method is described in Appendix C.

2.16 STATISTICAL TREATMENT OF DATA

In this thesis, n generally represents the number of different fruit samples tested. For example, in assays of enzyme activity, n = the number of extracts prepared from different fruit samples received at a certain physiological stage (ripening, ripe, unseparated, separated, etc.); for ethylene production n = the number of different fruit analysed from each consignment received. However, for the uronic acid estimation, n = the number of assays of each sample tested; and in the Feulgen staining, n = the number of nuclei examined.

Each sample assayed for enzyme activity, either by the cyanoacetamide method for reducing groups or *p*-nitrophenol hydrolysed from the *p*-nitrophenylglycosides (as described in Section 2.6), was always in triplicate with a corresponding blank control in duplicate. Since the absorbance value of the replicates was always very close, no standard deviation was calculated for these intra-assay replicates. Thus, the estimate of enzyme activity is the mean of the test in triplicate less the mean of the control in duplicate. Viscosity assays were usually in duplicate with one set of controls for the experiment.

When the result of a single tissue extract is presented, it confirms the results of at least two previous experiments where the same relative answer was obtained.

Where there is a large variation in the individual values of a group and, hence, a large standard deviation, each measurement is given independently because the factors responsible for the biological variation are not known and pooling the data is, therefore, unsuitable. In all instances, however, the data obtained have been directly linked to a physiological parameter, such as the extent of separation, stage of ripening or ethylene production. For most measurements, the mean and standard deviation for the sample has been calculated. The standard deviation is a sample measure of the dispersion of actual values from a mean. The standard error has not been computed since very little is known about the population as a whole, and the standard deviation was deemed best to show the variation in the sample measured.

A

B

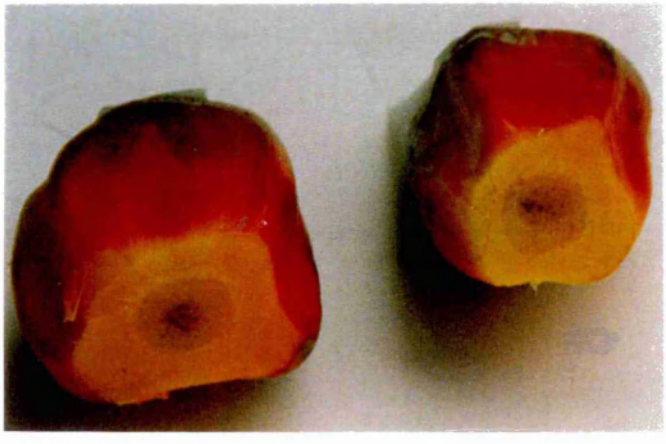


FIG. 3.1: RIPE KLUANG MUTANT FRUIT (209 DAA)

A. A spikelet with no apparent external signs of damage.

B. Transverse section of fruit. There is a darker ring of tissue in the centre of the fruit around the kernel which is never observed in normal fruit.

CHAPTER 3

SOME FEATURES OF NORMAL AND MUTANT NON-ABSCINDING OIL PALM FRUIT DURING RIPENING AND ABSCISSION

3.1 RIPENING IN NORMAL AND MUTANT FRUIT

3.1.1 The Appearance of the Fruit during Ripening

Figure 1.3 (page 29A) shows the appearance of unripe, ripening and ripe fruit on spikelets of the normal clone 271D. It can be seen that as ripening progresses the purple anthocyanins decrease (Fig. 1.3B, compare with Fig. 1.3C) and carotene accumulates (Fig. 1.3D) so that, with the exception of the area below the stigma, fully ripe normal fruit are bright orange at about 150 daa (Fig. 1.3E). However, after harvesting, unripe and ripening spikelets which separate after some days show no signs of increased carotene synthesis - they do not become orange or "ripen". Instead, fruit dehydrate around the abscission zone and also begin to "brown" starting at the fruit base and progressing towards the stigma (see Fig. 4.8, page 112A).

The fruit of the mutant non-abscinding palm DxP5 (Kluang Malaysia) appear to ripen like normal fruit. Fig. 3.1A shows a spikelet of undamaged ripe fruit from this palm which is 209 daa. However, in some fruit of a similar age, the outer fruit of the bunch, which are the terminal fruit of individual spikelets, show signs of rotting, as shown in Fig. 3.2A (210 daa). The mesocarp of these fruit has split open and fungal growth is seen on the exocarp. Also, the abscission zone appears rotten, but separation has not occurred at Position 2/3 (around the periphery of the fruit base). Pressure on the fruit has been applied (it has snapped at Position 2/3) in order to observe the zone (Position 1). Figure 3.3A shows that some fruit separate 1+4/5 because they rot off (see Appendix D, page 227, for comments by plantation field staff in Malaysia). Figure 3.3C shows a longitudinal section of a rotten fruit but no separation has occurred at the zone.

A transverse section of undamaged fruit (209 daa, Fig. 3.1B) shows a small dark ring around the kernel. In damaged fruit of about the same age (210 daa, Fig. 3.3B) the the mesocarp has become browny-orange and the central dark ring appears more prominent. This suggests that the deterioration starts from within the fruit initially around

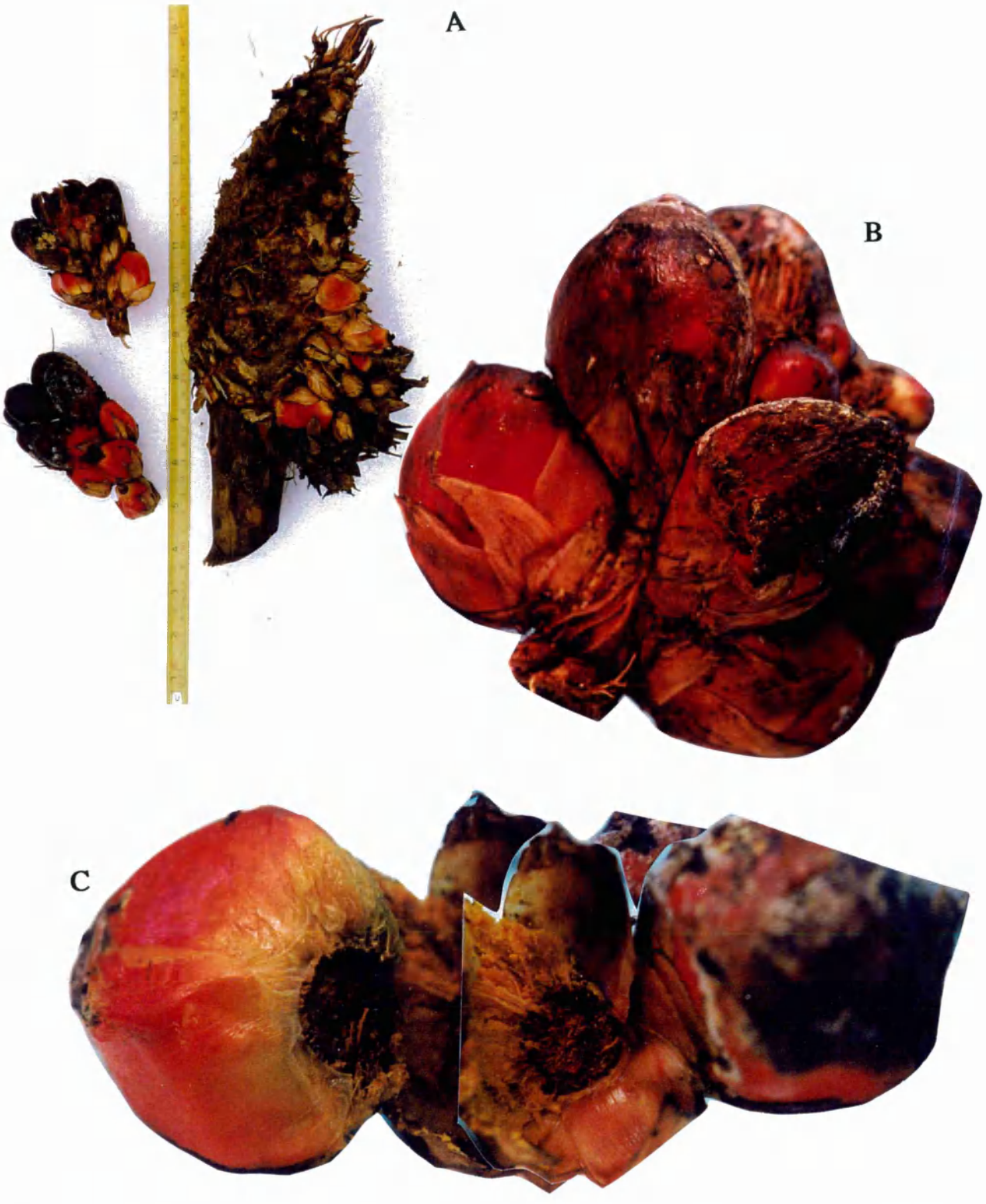


FIG. 3.2: RIPE KLUANG MUTANT FRUIT

A. A ripe fruit bunch (206 daa) with the majority of spikelets (~100) removed. The terminal fruit of the spikelets are damaged (each spikelet weighs ~110g; the whole fruit bunch, 12.2kg).

B. A spikelet with damaged fruit (210 daa) showing external fungal contamination.

C. An unseparated fruit is forced apart. No separation has occurred at the periphery (Position 2/3) and the abscission zone is discoloured.

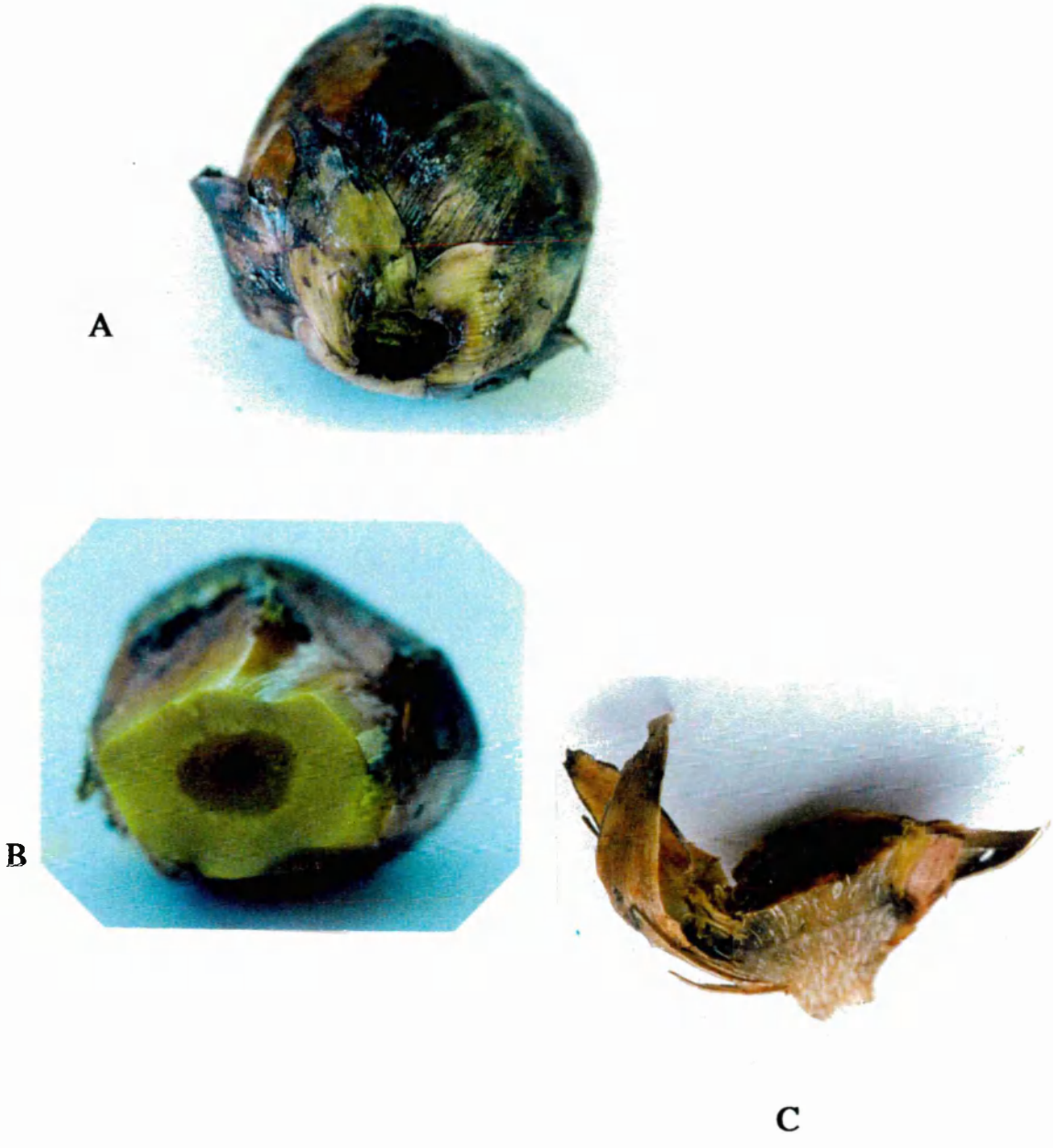


FIG. 3.3: KLUANG MUTANT FRUIT

A. A fruit (210 daa) which has finally detached from the spikelet due to rotting. No separation has occurred at Position 2/3 and it has rotted at Positions 1, 4 and 5.

B. A transverse section of a fruit with external damage. The mesocarp is browny-orange and the central ring of tissue is somewhat darker than in Fig. 3.1B.

C. A longitudinal section of the abscission zone of an unseparated damaged fruit. Separation has not occurred at any position.

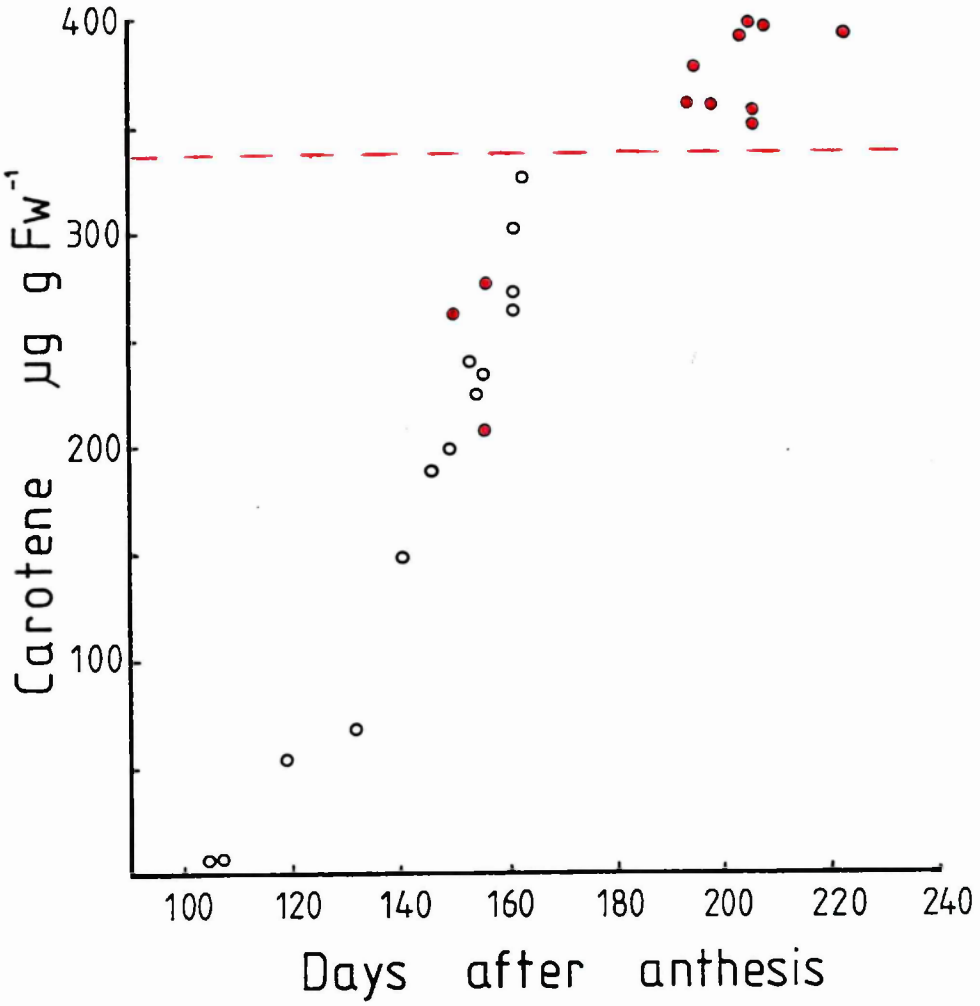


FIG. 3.4: CAROTENOID CONTENT OF THE KLUANG MUTANT MESOCARP COMPARED WITH THAT IN NORMAL FRUIT

Carotene was estimated in normal fruit (o) from 105-160 daa and in the Kluang mutant fruit (•) from 150-233 daa (by A. Norville, as described in Appendix C).

the fibre bundles surrounding the kernel and then progresses outwards until the fruit show visible signs of rotting.

Mesocarp carotene of clone 271D has been measured in our laboratory (extracted and assayed by A. Norville). Carotene levels increased from 10μg g Fw^{-1} in unripe normal fruit (105 daa) to 330μg g Fw^{-1} in ripe normal fruit (160 daa). The mutant non-abscinding palm DxP5 (Kluang, Malaysia) does not appear to have delayed ripening when carotene levels are compared with normal fruit. Non-abscinding mutant fruit of 150 daa and 156 daa have carotene levels similar to normal fruit of the same age (263μg g Fwt^{-1} and 212μg g Fwt^{-1} respectively). However, carotene accumulation in the mesocarp of the non-abscinding fruit continues to about 400μg g Fw^{-1} and 9 samples analysed between 194 daa-233 daa had a mean carotene level of 375.7 $\pm$ 19.9μg g Fw^{-1}. These results are shown in Fig. 3.4. The carotene level of the mutant mesocarp reaches a higher level than in ripe normal mesocarp.

Lipid accumulation also appears to occur as normal in the mutant palms. Analysis of percent oil of mesocarp by NMR (Unilever, Colworth House, Bedford) was as follows:

Clone 271D (140-155 daa)	=	77.0 $\pm$ 5.6	n = 14
Clones 926/924 (141-171 daa)	=	74.5 $\pm$ 3.4	n = 16
Kluang mutant (175-208 daa)	=	62.9 $\pm$ 6.4	n = 9

However, at the time of these mesocarp sample collections the deterioration that progresses from the kernel outwards in the Kluang mutant palm was not fully appreciated and a more rigorous sample collection and specific lipid analysis is required.

3.1.2 Ultrastructure of the Ripe Mesocarp

It always proved difficult to obtain satisfactory TEMs of ripe mesocarp. Unlike the avocado, for which good TEMs have already been published (Murray-Scott *et al*, 1963; Platt-Aloia & Thomson, 1981), oil palm mesocarp has a very high lipid content and contains many lignified fibres (Section 3.3) as shown in Table 3.1.

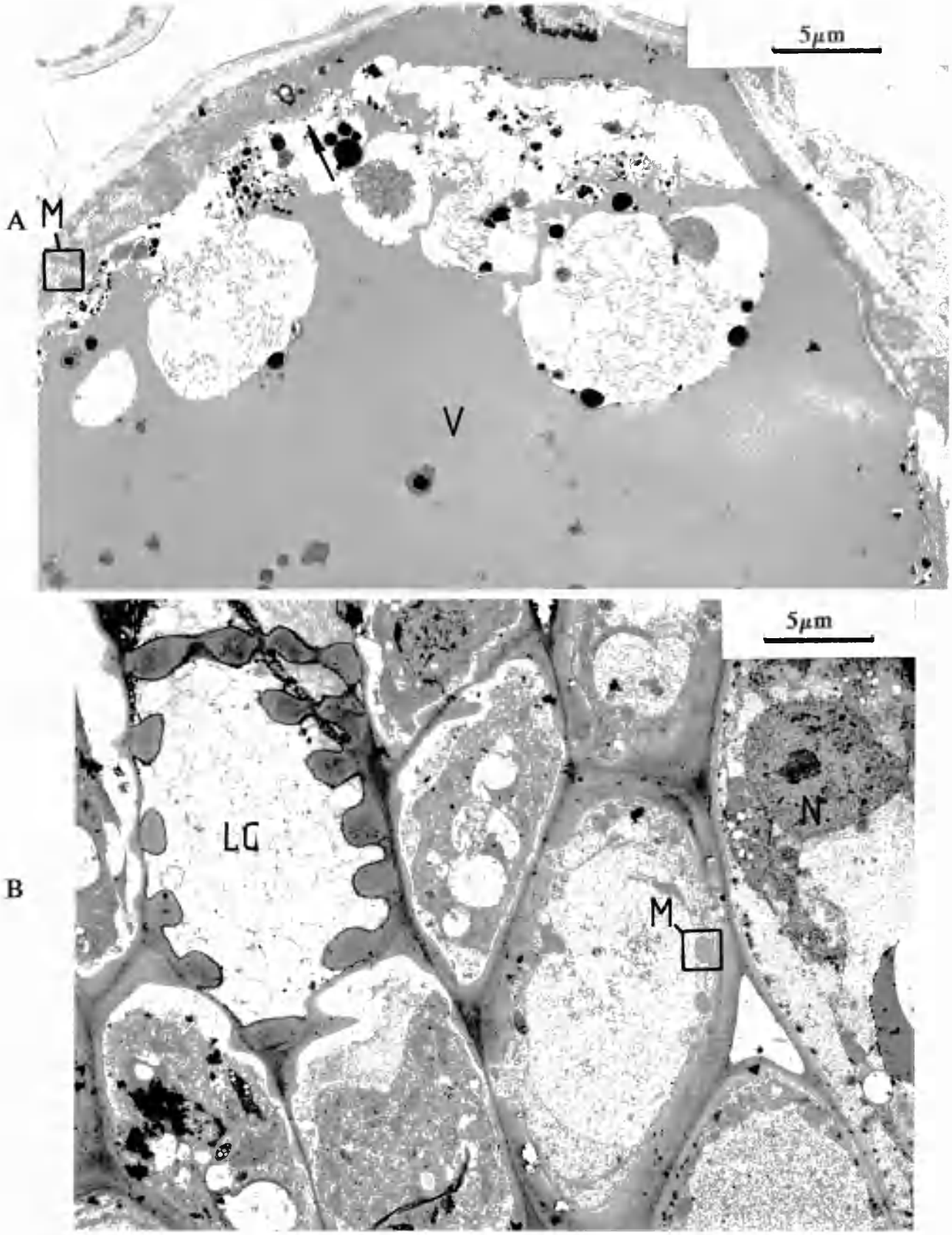


FIG. 3.5: TRANSMISSION ELECTRON MICROGRAPHS OF RIPE MESOCARP

A. An oil-containing cell (the arrow shows an area where there is loss of membrane integrity).

B. A lignified cell (LC) and adjacent parenchyma cells.

N = Nucleus; M = Mitochondrion; V = Vacuole.

	RIPE MESOCARP		
	Avocado	Oil Palm	
Lipid	11-39%	45-60%	35-60%
Fibre	2%	22-28%	11-21%
Water	52-79%	22-28%	-
Reference	Pearson (1975)	Wood (1981)	Hartley (1988)

Table 3.1 Comparison of the Lipid, Fibre and Water Content in the Ripe Mesocarp of Avocado and Oil Palm

When the embedded tissue for TEM is sectioned the hard fibrous material causes ripping and holes in the preparations. However, Ms H. Davies of The Open University Electron Microscopy Unit was able to produce some TEMs of ripe mesocarp. A large oil-containing cell is shown in Fig. 3.5A. The cell is highly vacuolated but there is loss of membrane integrity (see arrow). Parietal mitochondria are still intact. Cell types in the ripe mesocarp, other than oil-containing cells, are also observed in Fig. 3.5B, and comprise vascular tissue with adjacent parenchyma cells. There are regions of wall thickening seen in the xylem/fibre cell. The adjacent parenchyma contain numerous mitochondria. In contrast with the oil-containing cell, these cells have intact membranes - plasma membrane and tonoplast. In one cell, an intact nucleus with a large nucleolus can be seen. The cell wall thickness is variable and may be thinner in these mesocarp parenchyma cells than in the oil-bearing cells.

3.2 ABSCISSION OF NORMAL AND MUTANT FRUIT

3.2.1 Natural Abscission from the Palm

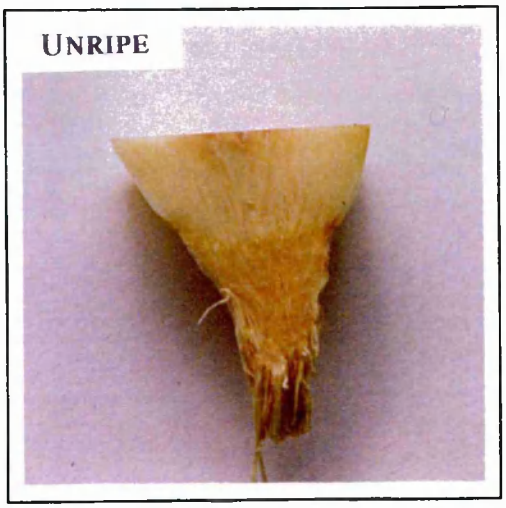
- **Normal Fruit**

The natural abscission of ripe fruit in normal wild type and clonal palms (including clones 271D, 90A, 926, 476G) has been discussed in Sections 1.3.2 and 2.2.1, and is shown in Figs. 1.8 and 1.9 (pages 35A and 35).

- **Non-Abscinding Mutant Fruit**

To date, four non-abscinding palms have been discovered. One palm, "Kluang non-abscinding DxP5" is on the Kluang plantation, Malaysia, and the other 3 palms,

A



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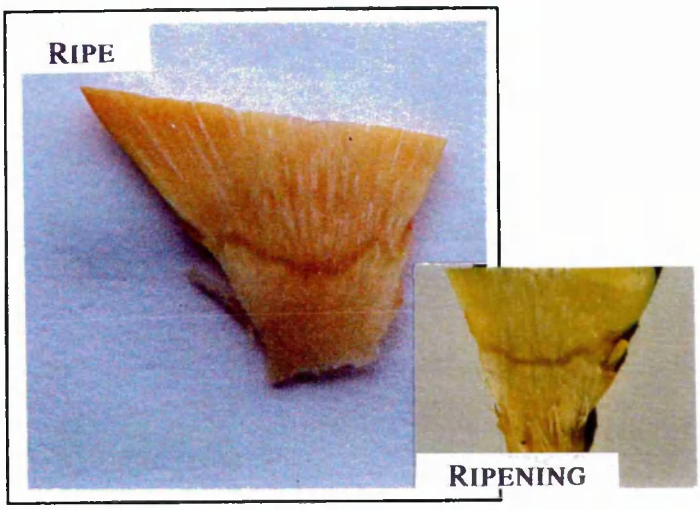

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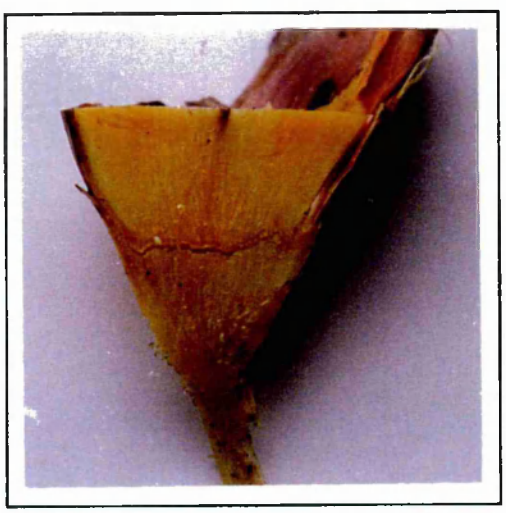


FIG. 3.6: LONGITUDINAL SECTIONS OF THE ABSCISSION ZONE IN UNSEPARATED AND SEPARATING NORMAL FRUIT

A. The abscission zone is barely visible in unripe fruit (57 daa).
B. Unseparated but translucent zones of ripe (145 daa) and ripening (137 daa) fruits.
C. Separation at Position 1 only in a ripening fruit (139 daa). The separation occurs at the lower edge of the translucent band, beginning in the centre of the abscission zone and spreading towards the fruit periphery.

Palms 1, 2 and 3, are at the Sabah plantation in Indonesia. Fruit on these palms is not shed at full ripeness. Even some fruit of bunches of the non-abscinding Kluang palm which have been harvested at 233 daa show no signs of separation. Fruit usually rot at the abscission zone and then fall to the ground with subtending RA and tepal whorls 1 and 2 still attached (Fig. 3.3A). That is, the fruit is released from the bunch after 1+4/5 separation. Field staff have reported that fallen fruit seen at the base of the non-abscinding Kluang palm are usually rotten. Occasionally, a few "good" fruit have been seen but even so, the mesocarp of these fruit is split and cracked. These fruit were thought to have been shed from the centre and bottom of the spikelets (mid and inner fruit). For Sabah Palm 1 a few fallen fruits were "good" but most were rotten and for Sabah Palm 2, all fallen fruits were rotten (see Appendix D, page 227).

In addition, Dr G. Smith of Unilever examined the non-abscinding Kluang Palm and, in particular, a fruit bunch of 184 daa. She reported that there was no sign of fruit abscission after the fruit on the bunch were "tweaked" with some pressure. There were no loose fruit on the bunch and no fallen fruit on the ground. This bunch was harvested two days later and sent to our laboratory in Oxford.

3.2.2 Post-Harvest Separation of Fruit

- **Normal**

The post-harvest separation of normal fruit has been described in Section 1.3.2 and 2.2.2. The type of separation observed is shown in Figs. 1.8-1.10 (pages 35A-36A) and Figs. 1.7 and 2.1 (pages 34A and 44A). Longitudinal sections of ripe unseparated fruit abscission zones show that, just prior to abscission, the zone (which normally cannot easily be seen by eye, see for example an unripe unseparated fruit, Fig. 3.6A) becomes visible as a translucent band of about 1mm wide (Fig. 3.6B) The appearance suggests a gelatinisation of pectins has occurred. The fruit is still firmly attached to the pedicel when the zone is translucent and cannot be removed even with great force. Figure 3.6C shows that separation at Position 1 occurs at the lower edge (pedicel side) of the translucent band, and this translucency is also seen in Figs. 1.7 and 2.1 (pages 34A and 44A). If force is applied to remove the fruit when it has only partly separated at the zone, it will fracture longitudinally through the mesocarp (see Fig. 3.9); it does not fracture transversely through the translucent zone which indicates that the zone (even when translucent) is not a site of cell wall weakness.

D

FIG. 3.6D: LONGITUDINAL SECTION OF THE ABSCISSION ZONE IN UNSEPARATED KLUANG MUTANT FRUIT (153 DAA)

The fruit were examined daily by longitudinal section for zone translucency. At 96h (4d) after arrival in the laboratory the abscission zone was beginning to show some translucency.

- **The Non-Abscinding Kluang Mutant**

The non-abscinding Kluang fruit arrived in the laboratory 24-30h after harvesting whilst Sabah fruit arrived about 48h post-harvest (an extra day was involved because the fruit had to be shipped from Indonesia to Singapore, then Singapore to the U.K.).

The ripe fruit of the non-abscinding Kluang and Sabah Palms 1, 2 and 3, did **not** shed whilst on the palm but **after** harvesting they all acquired the ability to separate. The fruit of Sabah Palms 1 and 3 separated between 24h and 48h in the laboratory; whereas the Kluang and Sabah Palm 2 non-abscinding mutants could take up to a week before this took place, and some terminal (outer) fruit did not separate at all. In the Kluang and Sabah 2 palms, it was noticeable that when fruit did separate the amount of free abscission zone cells that could be removed from the fruit side and pedicel side was less in weight than that obtained from normal separated fruit zones. For example:

On Arrival, 0h	Fruit Side (FZ)	Pedicel Side (PZ)
Normal 271D, 60 fruit	1.35g	0.38g
Kluang Mutant, 60 fruit	0.43g	0.28g

Table 3.2: **Comparison of the Amount of Tissue Obtained from the Separated Zone of the Normal and Kluang Mutant Fruit**

Twenty-seven different fruit consignments of the non-abscinding Kluang palm have been received (138 daa to 233 daa). Of these, 17 consignments consisted of the whole bunch either as individual spikelets or the intact fruit bunch. Thus, it is estimated from the number of spikelets received that about 12,000 Kluang mutant fruit were examined. Only a maximum of 50 fruit separated in transit (0.4%). On arrival (0h), a total of 700 (6%) had separated at Position 1, the majority of which could be detached only with some pressure (1+3) - these were the central and inner fruit of the bunch, rather than the outer (terminal) fruit which often had damaged mesocarp and some fungal contamination (Fig. 3.2A).

The Kluang bunch of 184 daa, which had been shown to have no fruit abscission before harvesting (examined by Dr G. Smith, Section 3.2.1), had some fruit separation when received in Oxford (3 days later). These were again the central and inner fruit. Although these fruit had separated at Position 1, they had not fully separated at Positions 2/3, so that pressure was required to detach the fruit indicating that complete separation at 2/3 had not occurred.

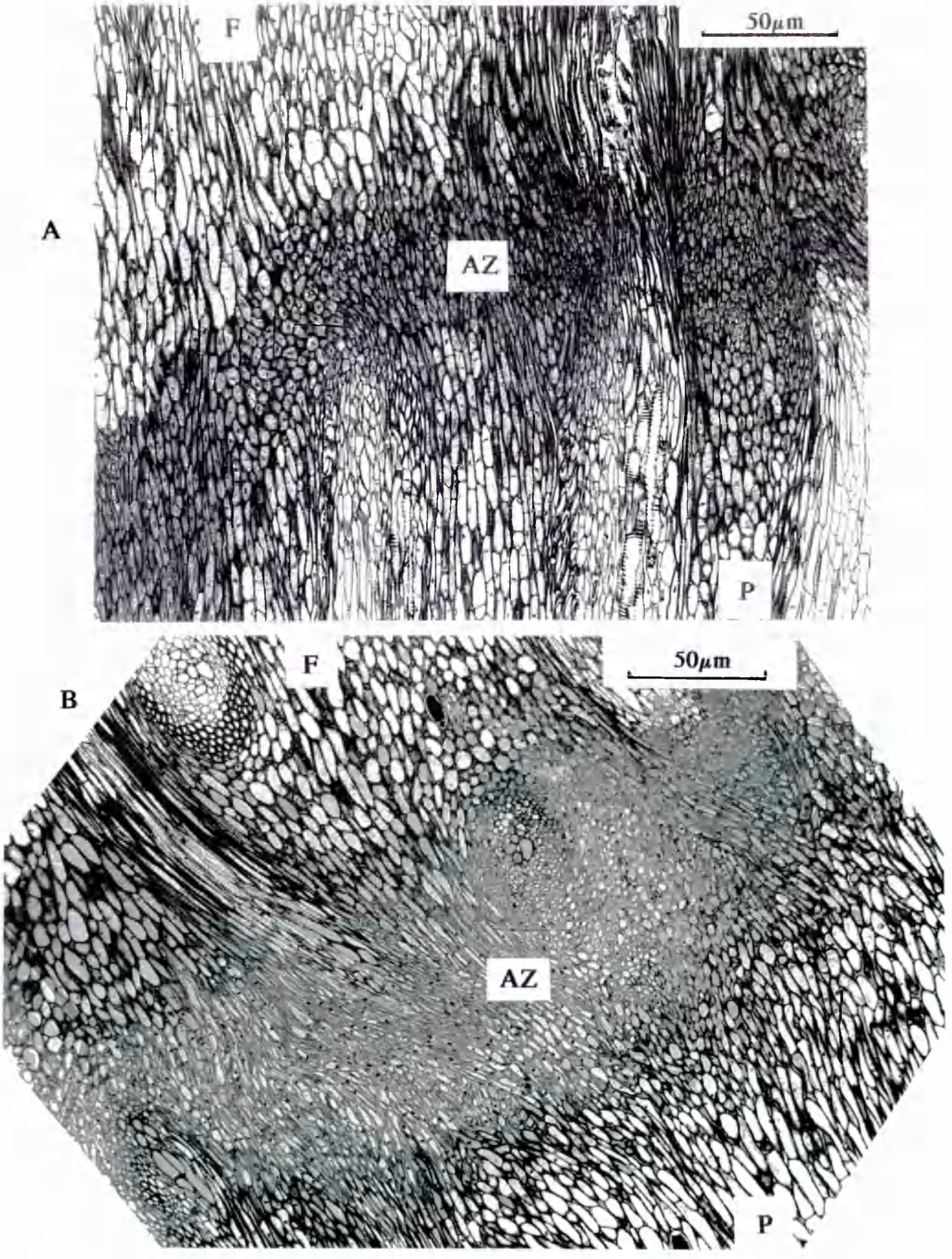


FIG. 3.7: LIGHT MICROGRAPHS OF UNSEPARATED ABSCISSION ZONE TISSUE

A. A longitudinal section of the unseparated abscission zone in ripening fruit (138 daa).

B. A longitudinal section of the unseparated abscission zone in ripe fruit (146 daa).

AZ = Abscission zone; F = Fruit side; P = Pedicel side.

On arrival in the laboratory, the majority of the unseparated zones examined in ripe Kluang mutant fruit were **not** translucent. However, the zone did become translucent (Fig. 3.6D) as a prelude to separation (as observed in normal fruit, compare Fig. 3.6A with Fig. 3.6B). Close examination and sectioning of unseparated fruit on arrival showed that whilst separation at Position 1 would occasionally have been initiated, no separation occurred at the fruit periphery (Position 2). The mutant Kluang non-abscinding palm is, therefore, not inherently incapable of abscission but does not achieve the process under normal conditions.

Eleven different consignments of fruit from Sabah Palms 1 and 2, and four from Sabah Palm 3 have also been received. Fruit sent from Sabah were a day longer in transit. As a result, several deliveries had extensive fungal contamination and had to be discarded (this rarely occurred with fruit samples from Kluang, although two or three consignments were badly infected). Sabah Palms 1 and 3 shed on arrival or within 24h. Thus, harvesting the spikelets of Sabah Palms 1 and 3 induces separation in a similar way to normal clones, whereas Sabah Palm 2 was more similar to the Kluang mutant with fruit that were very slow to separate.

3.2.3 Light and Electron Microscopy of Abscission Zones

Ms H. Davies of the Open University Electron Microscopy Unit has prepared sections of unseparated and separated abscission zones for light and electron microscopy. The sections were first observed by light microscopy (Fig. 3.7A-D). Ripening and ripe unseparated zone tissue is shown in Fig. 3.7A and B. Zone cells are smaller and mostly rounded in shape, compared with the larger more elongate cells of the mesocarp above and in the pedicel tissue below the zone. Vascular tissue (with spiral secondary thickening) becomes attenuated as it passes through the zone (Section 3.3) and although no continuous vascular bundles are seen in the sections, their path can be traced. Figure 3.7C shows a section of a ripe fruit just separating at Position 1 and Figure 3.7D shows the fully separated pedicel side of the zone. Intact individual cells also are observed as in other abscission systems (*Sambucus nigra*). The oil palm abscission zone does not comprise 1-2 layers of cells only (as in the bean, *Phaseolus vulgaris*) but is multicellular and, unlike bean, these cells do become loose from each other. It is, therefore, possible to harvest them as distinct from non-zone tissue on either side.

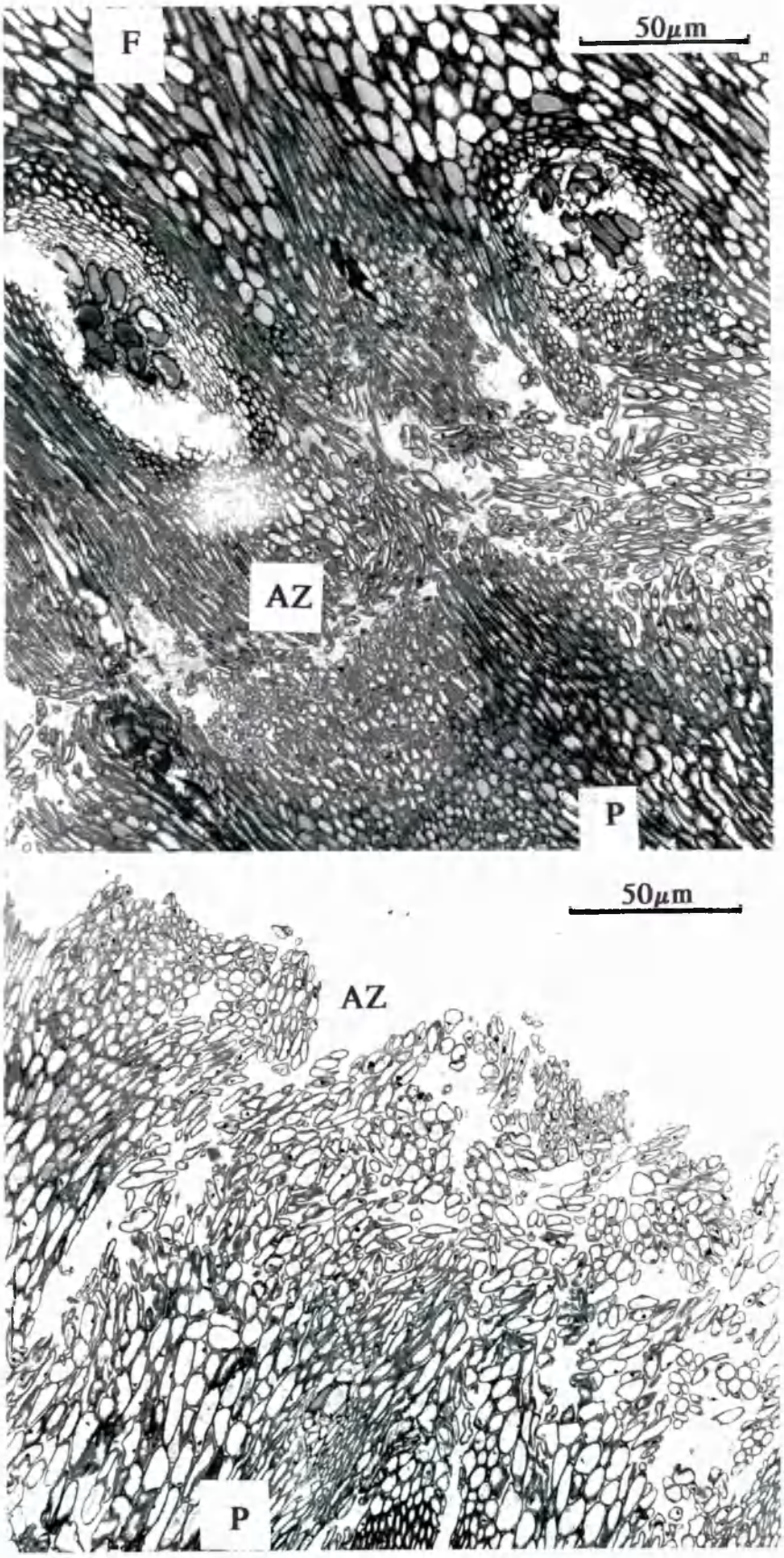
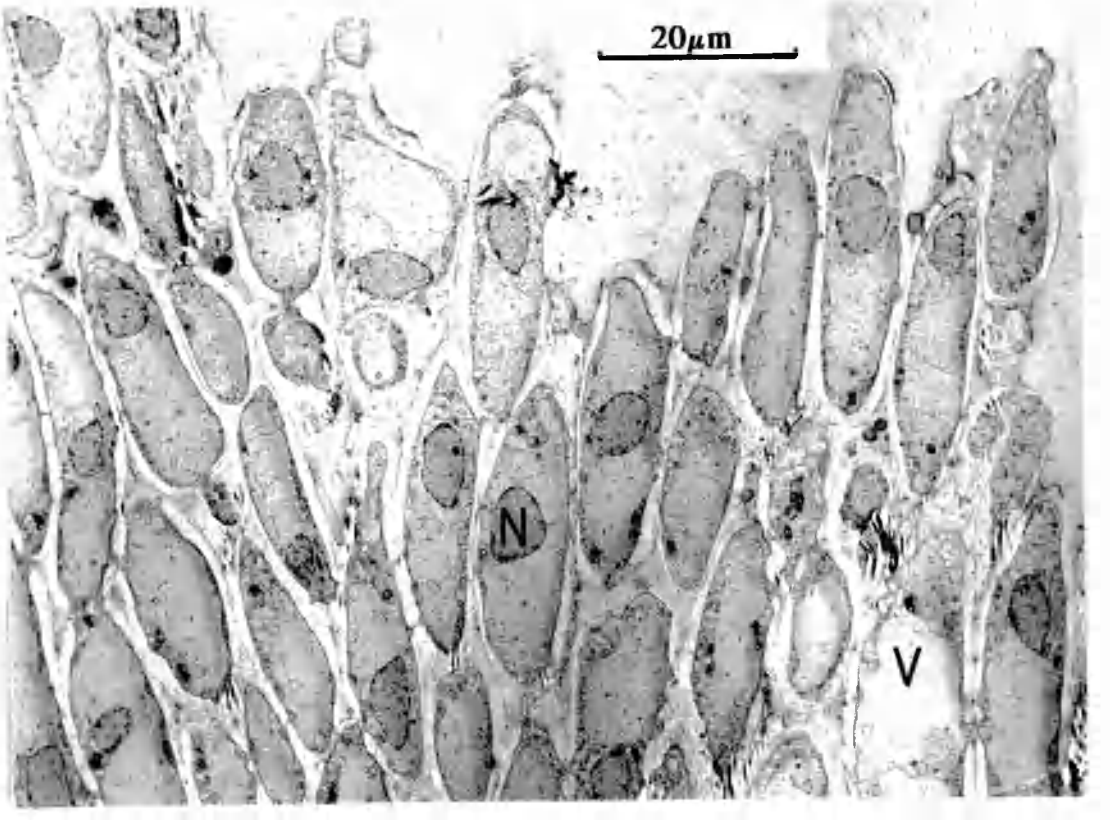


FIG. 3.7: LIGHT MICROGRAPHS OF SEPARATING/SEPARATED ABSCISSION ZONE TISSUE

C. A longitudinal section of the central region of a ripe abscission zone (146 daa) just starting to separate.

D. The fully separated pedicel side of the zone of a ripe fruit (160 daa) after natural fruit separation.



A

FIG. 3.8A: TRANSMISSION ELECTRON MICROGRAPH OF ABSCISSION ZONE CELLS FROM A NATURALLY SEPARATED RIPE FRUIT (160 DAA)

N = Nucleus; V = Vacuole.

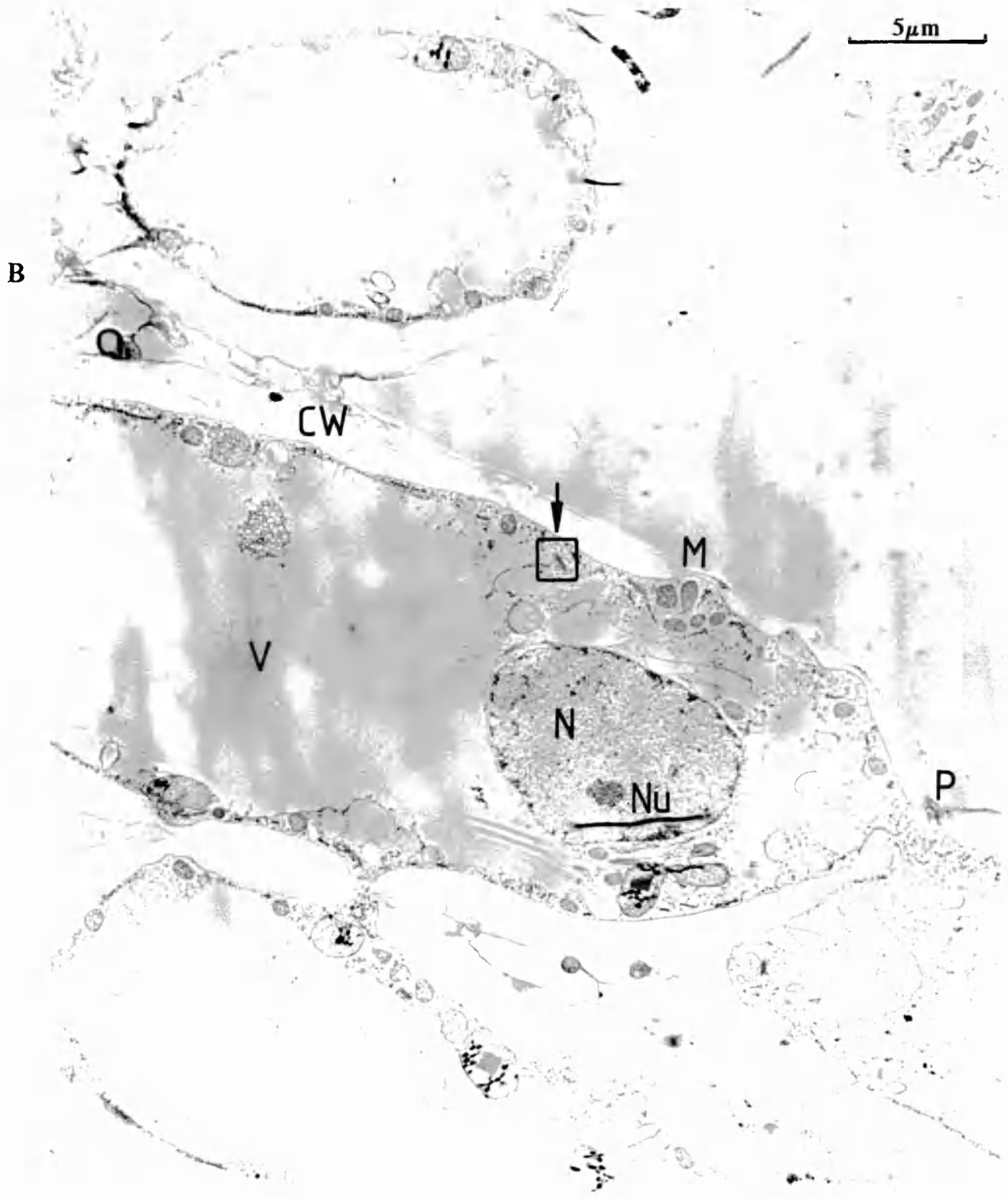


FIG. 3.8B: TRANSMISSION ELECTRON MICROGRAPH OF SEPARATED ABSCISSION ZONE CELLS

The arrow points to dictyosomes shown at a higher magnification in Figure 3.8C (opposite).

P = Plasmodesmal strands; M = Mitochondrion; N = Nucleus; Nu = Nucleolus; V = Vacuole; CW = Cell Wall.

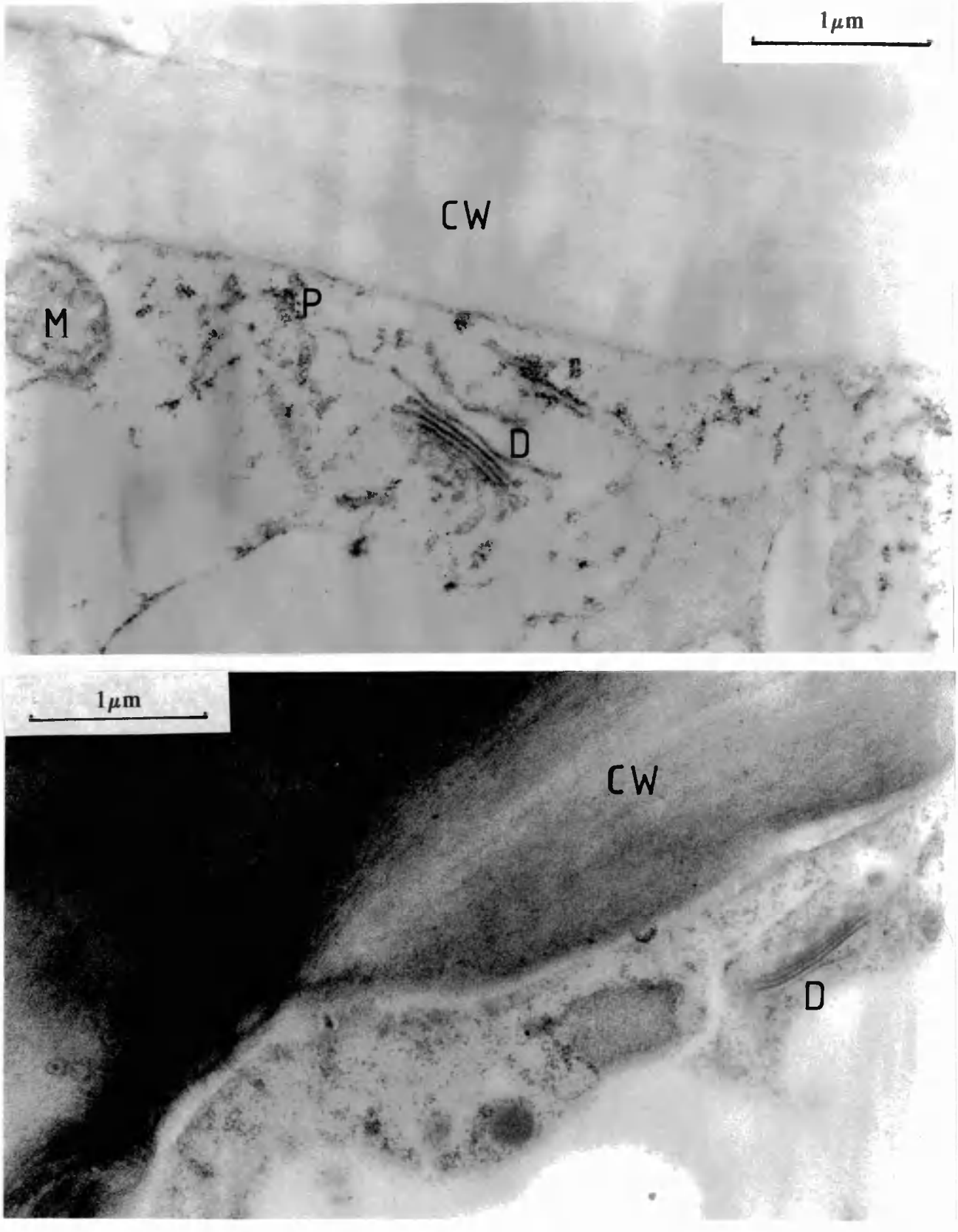


FIG. 3.8: TRANSMISSION ELECTRON MICROGRAPHS OF DICTYOSOMES IN ABSCISSION ZONE CELLS

C. From a separated zone of a ripe fruit (160 daa).

D. From an unseparated zone of a ripening fruit (138 daa).

P = Polysomes; D = Dictyosome stacks; M = Mitochondrion; CW = Cell Wall.

FIG. 3.9: FIBRES IN THE FRUIT MESOCARP

Complete cell separation at the abscission zone has not occurred so that some fruit tissue remains strongly attached to the pedicel. Thick fibre bundles can be seen passing longitudinally through the mesocarp and these then abut the zone (see Fig. 3.10).

The transmission electron microscopy of these separated abscission zone cells is shown in Figure 3.8A & B. The nuclei are large, the nuclear membrane is intact (Fig. 3.8B), and there appears to be active cytoplasmic activity. Both the plasma membrane and tonoplast are reasonably well preserved, which contrasts with the oil-containing cells of the mesocarp (Fig. 3.5A). In Fig. 3.8B, plasmodesmal strands can be seen connecting two cells. At higher magnification (Fig. 3.8C), an expanded dictyosome stack with associated vesicles is clearly visible, along with polysomes, suggesting secretory activity typical of cells undergoing abscission. As a comparison, Fig. 3.8D shows a non-active dictyosome from an unseparated zone cell (138 daa).

3.3 VASCULAR CONNECTIONS THROUGH THE ZONE

Vascular connections between the fruit and pedicel are distributed throughout the whole abscission zone (Fig. 3.9). Abnormal abscission behaviour could have an anatomical basis and be due, for example, to differences in the vascular connections passing through the zone.

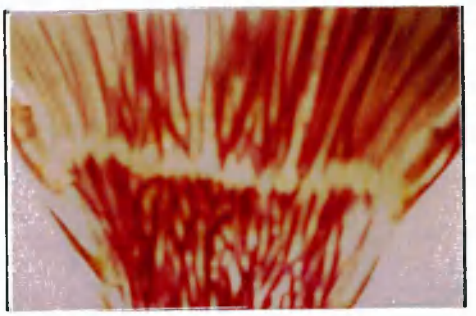
Longitudinal sections of unseparated ripe fruit of normal (271D) and the Kluang mutant non-abscinding palm were fixed and stained by phloroglucinol/HCl as described in Section 2.11.1. The lignified fibre elements of the pedicel below and the fruit mesocarp above abut the abscission zone cells (Fig. 3.10). The vascular traces which pass through and cross the tissue of the zone become extremely attenuated (Fig. 3.10D-I).

The amount of phloroglucinol/HCl stained vascular/fibre elements and non-stained parenchyma ground tissue was calculated as described in Section 2.11.1 for the zone, pedicel and mesocarp in both normal and Kluang mutant fruit. The phloroglucinol/HCl stained vascular/fibre elements as a percentage of the cross-section is shown in Table 3.3 (n = number of different longitudinal sections of fruit analysed).

	% PHLOROGLUCINOL/HCl-STAINED TISSUE ± Standard Deviation	
TISSUE	NORMAL 271D	KLUANG MUTANT
Zone	7.5% ± 2.5% (n = 14)	6.6% ± 2.4% (n = 9)
Mesocarp	47.9% ± 7.1% (n = 12)	40.7% ± 7.7% (n = 4)
Pedicel	52.4% ± 6.1% (n = 12)	43.2% ± 9.1% (n = 4)

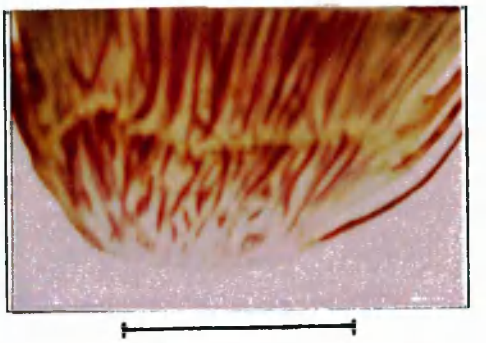
Table 3.3: The Percentage of Stained Vascular/Fibre Tissue of the Abscission Zone, Pedicel and Mesocarp Tissues

A
144 daa



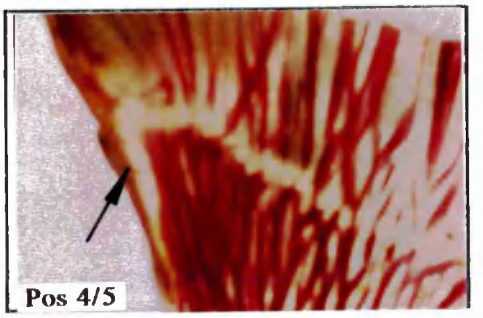
6mm

B
144 daa



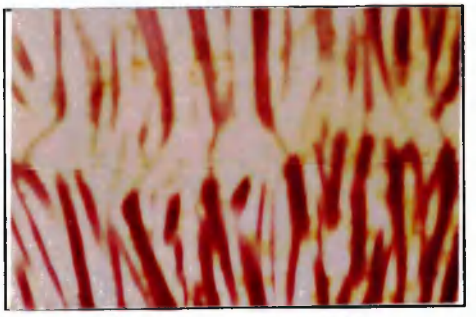
6mm

C
151 daa



4mm

D
144 daa

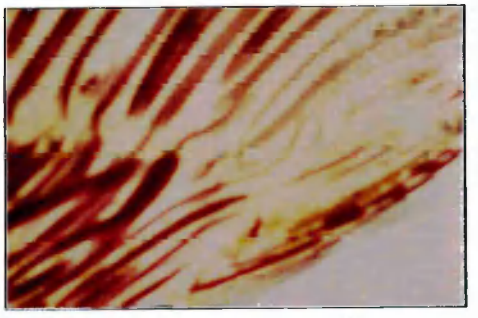


1mm

E
144 daa

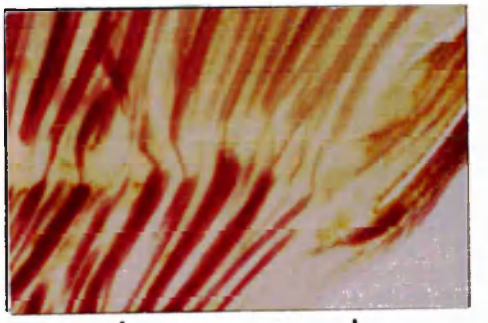
1mm

F
144 daa



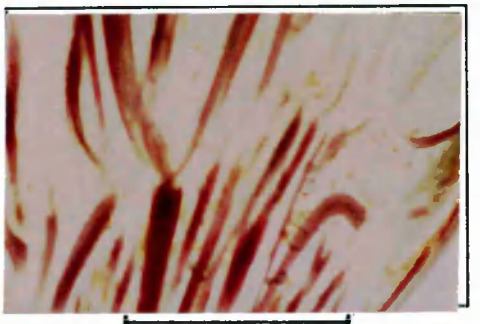
1mm

G
144 daa



1mm

H
151 daa



1mm

I
151 daa

1mm

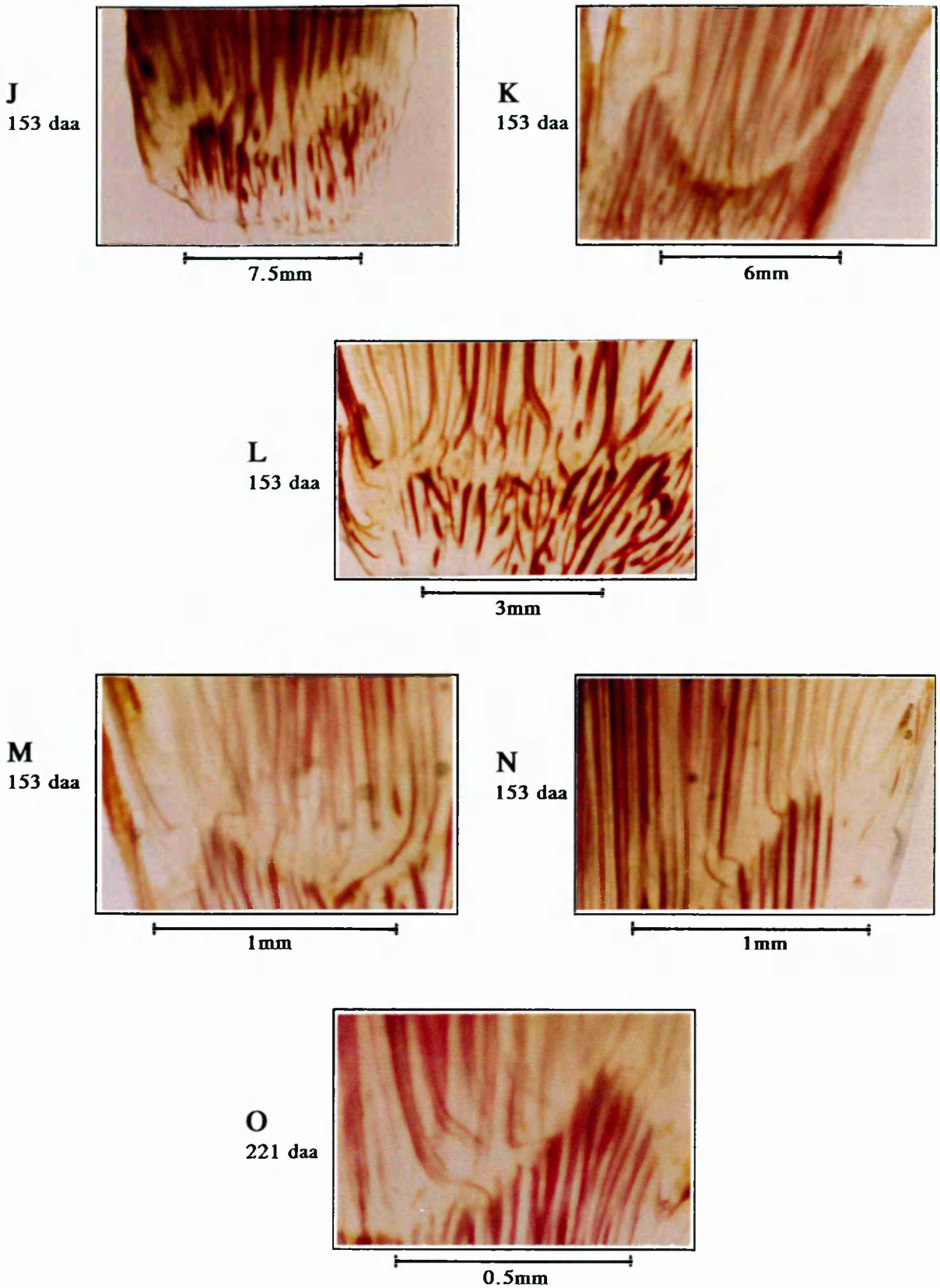


FIG. 3.10: LONGITUDINAL SECTIONS OF ABSCISSION ZONE TISSUE IN NORMAL AND KLUANG MUTANT FRUIT.

The sections were stained with phloroglucinol/HCl to show the lignified fibre tissue and attenuated vascular connections through the zone.

A-I. Normal fruit.

J-O. Kluang mutant fruit.

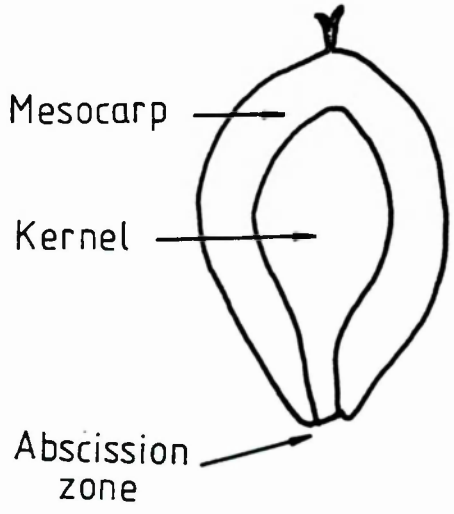


FIG. 3.11: THE KERNEL OF THE FRUIT

The kernel is encapsulated by fibres which project to the abscission zone. The actual size of the fruit and kernel is shown in the diagram.

The results show that there is no substantial difference in the amount of vascular tissue passing through the abscission zone of the normal (7.5%) and Kluang non-abscinding (6.6%) palms to account for failure of separation in the mutant, although the values for the pedicel and mesocarp in the normal are slightly higher than those in the Kluang mutant tissues.

The value of 40-48% calculated for the normal mesocarp tissue (Table 3.3) is higher than the overall fibre content reported by others for mesocarp. Values of 22-28% (Wood, 1981) and 11-21% (Hartley, 1988) represent the average fibre content throughout the mesocarp. The mesocarp analysed in these present experiments was directly above the zone and just below the kernel, where the fibres form a sheath around the inner kernel. Figure 3.11 shows this "sheath" of fibres firmly attached to the kernel, with a "stalk" of fibres projecting to the zone. This, then, may account for the higher percentage of lignified fibre tissue obtained for the mesocarp in this analysis.

The site where tepal separation occurs at Positions 4 and 5 is clearly seen in Fig. 3.10C and Fig. 3.10E-I shows that the few vascular connections crossing these positions also are attenuated, as in Position 1.

3.4 DNA CONTENT OF ABSCISSION ZONE CELLS

The DNA content of abscission zone (PZ) cells of separated fruit was determined as described in Section 2.11.3. The Feulgen stain methodology commonly uses hydrolysis in 1N HCl at 60°C for 8min. Abscission zone cells hydrolysed in this way showed only slight staining of nuclei and a reading of about 1.0 on the microdensitometer. Substitution of 1N HCl with 5N HCl has been recommended. An analysis of the optimum hydrolysis conditions gave the following results after the Feulgen stain.

Hydrolysis Conditions			Absorbance at 565nm ± Standard Deviation
1N HCl 60°C	10 min	(n = 27)	1.16 ± 0.50
5N HCl 60°C	10 min	(n = 37)	2.09 ± 0.90
5N HCl 20°C	15 min	(n = 30)	2.74 ± 0.82
5N HCl 20°C	60 min	(n = 33)	1.73 ± 0.62
5N HCl 20°C	120 min	(n = 22)	1.03 ± 0.37

Table 3.4: **The HCl Hydrolysis Values for Feulgen Staining of Abscission Zone Cells (n = number of different nuclei examined)**

Separated abscission zone cells hydrolysed in 5N HCl at 20°C for 15min gave maximum staining of DNA with the Feulgen reagent, with a mean absorbance value of 2.74.

The estimated DNA content per nucleus (pg) in abscission zone cells was determined using onion root tips as a standard. A widely accepted 2C value for onion is 33.5pg (Bennett & Smith, 1976). An absorbance of 31.30 ± 6.47 (n = 63) was obtained by microdensitometry for onion 2C nuclei.

$$\therefore \text{ pg DNA in oil palm abscission zone cells} = 2.74 \times \frac{33.50}{31.30} \text{ pg}$$

$$= 2.93 \text{ pg}$$

Jones *et al* (1982) have reported a 2C value for oil palm callus cells of 2.4 ± 0.4pg (relative to mouse lymphocytes) and 2.0 ± 0.3pg (relative to chick erythrocytes).

The closeness of this value for abscission zone cells of 2.9pg to the values of 2.4 ± 0.4pg and 2.0 ± 0.3pg of Jones *et al* (1982) for callus indicates that abscission zone cells are also 2C. No endoreduplication to 8C has occurred as in the squirting cucumber (*Ecballium elaterium*) fruitlet abscission zone (Wong & Osborne, 1978). In peach fruit abscission zone cells (Tirlapur *et al*, 1995) the DNA is assumed to be fragmented since a decrease in ethidium bromide fluorescence occurs. If DNA degradation is occurring in oil palm abscission zone cells, the Feulgen stain does not detect any decrease from the total DNA 2C value. In bean (*Phaseolus vulgaris*) also, no change in DNA content occurs during abscission of secondary zones (McManus *et al*, 1998).

3.5 PECTIN IN THE MESOCARP, PEDICEL AND ABSCISSION ZONE

Ruthenium red is a well known stain for the localisation of pectin in plant tissues (Chayen *et al*, 1969). When longitudinal sections of oil palm abscission zones were stained with ruthenium red, the whole section stained pink and showed no differentiation between the zone, mesocarp or pedicel. Pectin obviously was present in all tissues and so other methods were sought to visualise the polygalacturonate in pectin. In addition, ^{13}C CP-MAS NMR spectroscopy was used to determine any differences in the ratio of polygalacturonate to methylesterified-polygalacturonate (pectin).

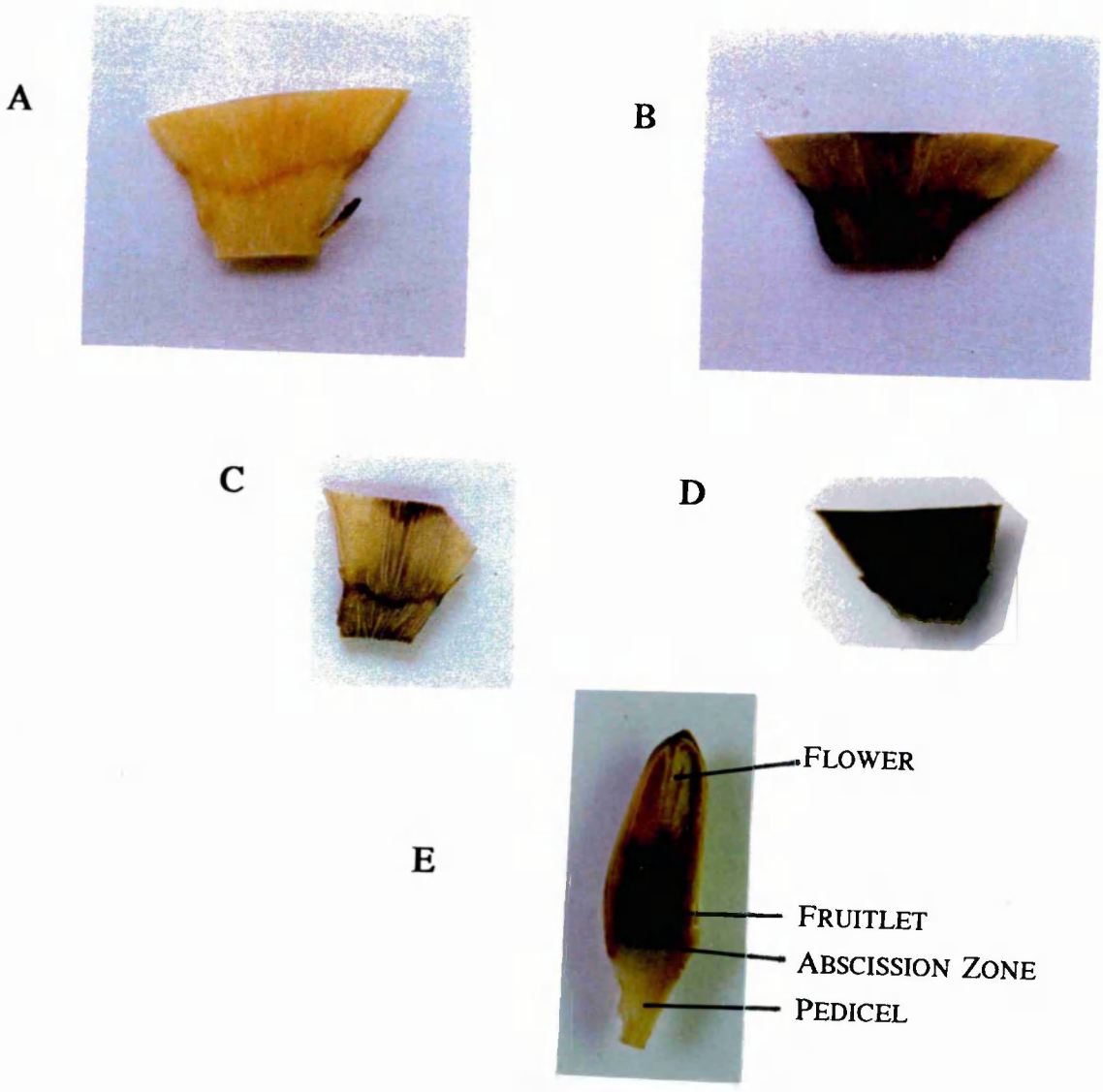


FIG. 3.12: LOCALISATION OF POLYGALACTURONATE BY Ni^{2+}/Na_2S IN LONGITUDINAL SECTIONS OF THE ABSCISSION ZONE

A. An unseparated ripe (145 daa) fruit with a translucent abscission zone.

B. A section of the same fruit as (A), stained with Ni^{2+}/Na_2S, showing a dark black precipitate in the abscission zone.

C. An unseparated unripe (118 daa) fruit. The abscission zone was not translucent but stained with a similar intensity as the ripe fruit with a translucent zone (A, B).

D. An unseparated unripe (63 daa) fruit. The mesocarp stains more intensely than older fruit (B, C) and the zone is not as clearly distinguished.

E. A pre-anthesis fruitlet showing some staining of the abscission zone (the section was only treated briefly with Na_2S, because the whole section blackened and the zone was then difficult to see).

3.5.1 Localisation of the Polygalacturonate Residues in Pectin

The method of Varner and Taylor (1989) was used as described in Section 2.11.2. The principle of this method is that Ni^{2+} or Co^{2+} can replace Ca^{2+} and bind to uronic acids. Thus, polygalacturonate-rich areas in pectin can be visualised before and after separation, and abscission zone pectin can be compared with that in the tissues above (mesocarp) and below (pedicel fibres).

Figure 3.12A shows a longitudinal section of a translucent, unseparated, abscission zone of a normal ripe fruit (145 daa) and another section of the same fruit (Fig. 3.12B) stained for polygalacturonate. An intense black nickel sulphide precipitate at the abscission zone is visible. There is also some staining of the mesocarp and pedicel tissue (but not the fibres). Since the pectin of the zone bound the Ni^{2+} ion to a greater degree than in mesocarp and pedicel, and formed an intense black precipitate with sodium sulphide, this indicates that the zone is composed largely of polygalacturonate rather than methylesterified pectin. When longitudinal sections were pre-treated with bleach, to demethylate the pectin to polygalacturonate, the whole section turned black. This also suggests that the pectin present in the mesocarp and pedicel is more highly methylesterified than in the abscission zone. In addition, very young mesocarp tissue (from pre-anthesis to 90 daa) stained much more intensely with Ni^{2+}/Na_2S than in ripening and ripe mesocarp (Fig. 3.12D and E). Perhaps the pectin of young fruit (which are developing and increasing in size) is less esterified than when the fruit has reached its maximum size and starts to synthesize lipid (see Section 3.5.3).

The translucent zone appears as if gelatinisation of pectin has occurred. The gelling ability of partially esterified pectin in solution is sensitive to the degree of esterification and Ca^{2+} concentration (Thibault & Rinaudo, 1986). Therefore, just prior to separation, activation of pectin methylesterase(s) in the abscission zone might demethylate the pectin to form the pectin gel, the zone then appearing translucent. As Fig. 3.6A shows, the young unripe, unseparated abscission zone is barely visible, and, thus, the pectin of the zone may then be more highly methylesterified. To determine this, it was necessary to stain **non-translucent** zones of **unripe** fruit for polygalacturonate. Figure 3.12C shows that the non-translucent zone in unripe unseparated fruit (118 daa) does stain with a similar intensity as the translucent ripe zone (Fig. 3.12B). This indicates that the transformation of the zone from the non-translucent stage to the translucent stage

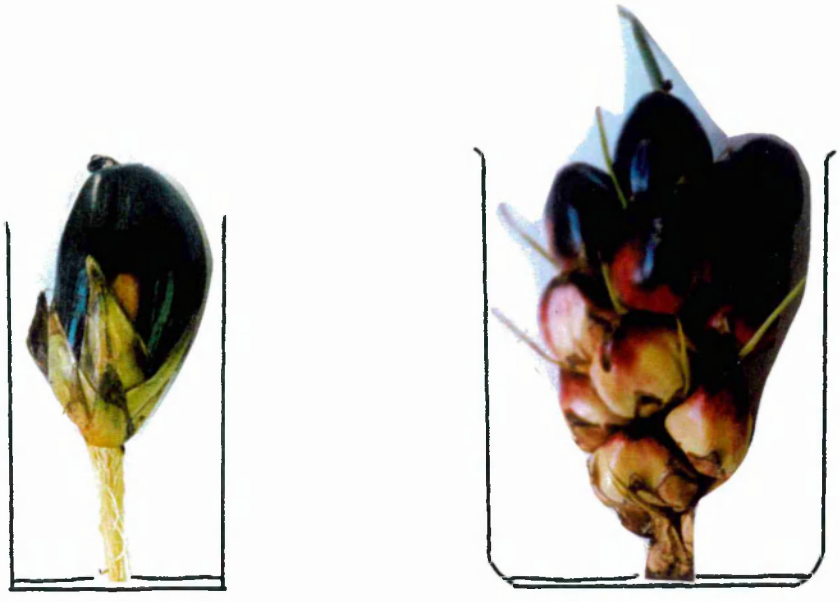


FIG. 3.13A: METHOD OF TREATING INDIVIDUAL FRUIT AND WHOLE SPIKELETS WITH TEST SOLUTIONS

FIG. 3.13B: AN UNSEPARATED RIPENING FRUIT (136 DAA) FROM A SPIKELET TREATED WITH AN EDTA SOLUTION

The fruit is still unseparated 72h after treatment with EDTA, and a longitudinal section shows that the zone is translucent.

probably does not involve extensive demethylation by pectin methylesterase(s), and that the walls of the abscission zone cells are rich in polygalacturonates from early in abscission zone differentiation. Even the pre-anthesis fruitlet, which already has a differentiated abscission zone (Henderson & Osborne, 1990), appears to be rich in polygalacturonates (Figure 3.12D).

The presence of unesterified polygalacturonate in the abscission zone cell wall is also suggested by the effect of calcium ion. Polygalacturonate can bind Ca^{2+} to form eggbox structures of dimers and larger aggregates (Carpita & Gibeaut, 1993). The effect of $CaCl_2$ and EDTA on abscission was tested by placing individual fruits in glass vials with their pedicel fibres in ~500μl-1ml of solution (Fig. 3.13A). Whole spikelets were placed in beakers (250ml) with 5-10ml of solution. When individual fruits (139 daa, n=10) were treated with a solution of 1M $CaCl_2$ (15h) separation was delayed for up to 48h (Table 3.5). This experiment was repeated with intact spikelets (136 daa) and the same result was obtained.

	% SEPARATION		
	48h	72h	96h
1M NaCl or DI H_2O	100%	-	-
1M $CaCl_2$	10%	20%	70%
0.5M EDTA, pH 6.0	0%	20%	80%

Table 3.5: Delayed Separation of the Abscission Zone with Calcium and EDTA Treatments

It was thought that the EDTA treatment would chelate Ca^{2+} in the cell walls of the abscission zone causing dissociation of pectin chains and speeding separation (Table 3.5). However, it delayed separation considerably; it was later found that EDTA strongly inhibits polygalacturonase activity in the oil palm (see Section 6.1.4). Figure 3.13 shows that the EDTA treatment did not prevent the zone from becoming translucent.

3.5.2 Uronic Acids in Cell Walls of Mesocarp, Pedicel and Abscission Zone

The amount of pectin (as uronic acid), whatever the degree of methylesterification, was compared in the mesocarp directly above the zone, pedicel and zone by the method described in Section 2.12. Three different separated zones were tested. Ripe separated zone (152 daa), the FZ/PZ tissue oven-dried before solvent extraction (Ripe Zone 1); ripe

zone tissue (152 daa) collected from freshly separated FZ/PZ and **not** oven-dried before solvent extraction (Ripe Zone 2); zone from ripening fruit (124 daa) which separated after 3 days (72h) and the FZ/PZ tissue oven-dried before solvent extraction.

		Galacturonic Acid Equivalents ± Standard Deviation	
Tissue	**n**	**mg g^{-1} Dry Weight**	**μmol mg^{-1} Dry Weight**
Mesocarp directly above zone	8	209.2 ± 17.0	0.99 ± 0.08
Pedicel	7	172.9 ± 10.9	0.82 ± 0.05
Ripe Separated Zone 1	4	274.9 ± 34.4	1.30 ± 0.16
Ripe Separated Zone 2	4	322.1 ± 25.9	1.52 ± 0.12
Ripening Separated Zone	4	339.9 ± 43.3	1.60 ± 0.20

Table 3.6: The Uronic Acid Content of the Mesocarp, Pedicel and Abscission Zone Tissues Estimated with the *m*-hydroxydiphenyl reagent (Blumenkrantz & Absoe-Hansen, 1973); (n = number of assays of the same sample)

The separated zone tissue, whether ripe or ripening, appears to have a similar uronic acid concentration, confirming the histochemical staining by Ni^{2+}/Na_2S (Section 3.5, Fig. 3.12). In addition, the uronic acid concentration in the zone was, on average, 49% greater than in the mesocarp and 80% greater than in pedicel tissue. The phloroglucinol/HCl staining of the fibre-xylem elements (Section 3.3) shows that the mesocarp and pedicel tissues both contain many more lignified fibre elements than the zone. These lignified fibre elements would not be expected to contain large amounts of uronic acid.

3.5.3 Solid State ^{13}C NMR of Mesocarp, Pedicel and Abscission Zone

Direct observation of the cell wall structure by solid state ^{13}C CP-MAS (Section 2.13) was carried out with Dr Stephen J. Heyes of the Inorganic Chemistry Laboratory, University of Oxford.

The histochemistry described in Sections 3.3 and 3.5.1 show that both lignin and pectin is present in the oil palm mesocarp, zone and pedicel tissues. Other substances also comprise these heterogeneous tissues, such as cellulose, hemicellulose, protein and lipid. However, it is the signals from the ^{13}C of the **major components** in each tissue which are observed in the ^{13}C CP-MAS NMR spectra.

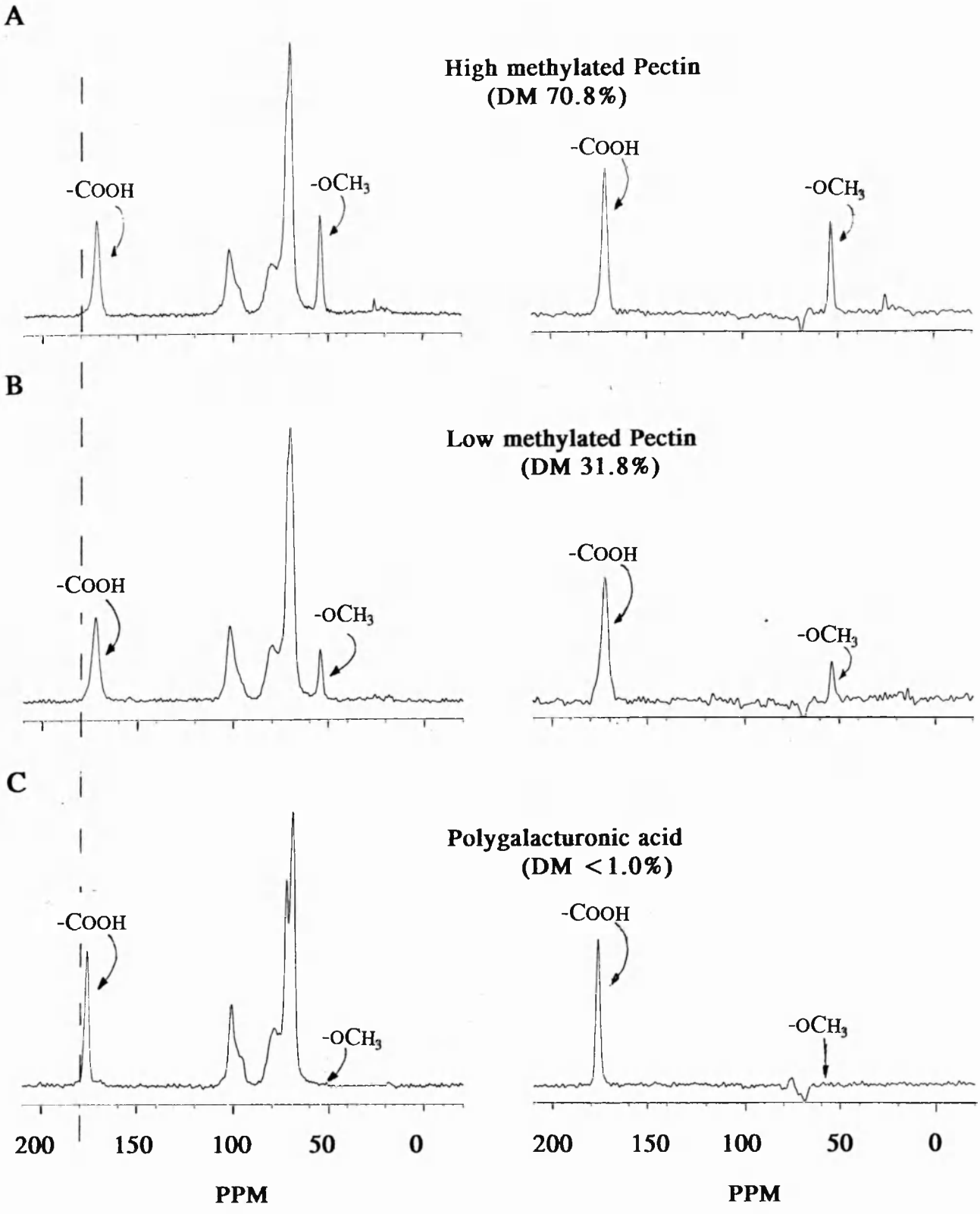


FIG. 3.14: ^{13}C CP-MAS NMR SPECTRA OF HIGH, LOW AND DEMETHYLATED (POLYGALACTURONIC ACID) PECTIN

The spectrum on the left is of all ^{13}C resonances and on the right, an NQS-Delay spectrum. The resonance of the C-6 of methylesterified galacturonan is at ~171 ppm (A, B) and at ~176-177 ppm in galacturonate potassium salt (C) (the broken line is at 180 ppm). The intensity of the resonance for the methoxyl carbon at ~54 ppm reflects the degree of methylation in the pectin, and is absent in polygalacturonic acid.

Although some comparisons can be made with other ^{13}C NMR studies of plant cell walls (oil palm leaf and trunk cell walls have been examined by Gallacher *et al* (1994) and Jarvis (1994)), it is often not possible to be specific about signal assignments because functional groups in a particular spectrum can vary by a few ppm. Substituent effects, the local environment of the functional groups, the purity of the sample, and the experimental conditions (e.g. the amount of water in the sample) all can affect the position of the peaks in the spectrum. In general, the chemical shifts of the ^{13}C in the carboxyl group of esters and acids (e.g. galacturonan, fatty acids and triacylglyceride) is between 155-185 ppm; in aromatics between 115-165 ppm; carbons in carbohydrate (cellulose, xylan, galactan, etc.) between 60-110 ppm; and in methylene and methyl groups (e.g. fatty acids) between 0-50 ppm (Williams & Fleming, 1980; Ha *et al*, 1996).

In this analysis, to help confirm the chemical shift of the carboxyl (C-6) and methoxyl groups in galacturonan, pectin standards with a known degree of methylation were analysed under the same conditions. Two spectra are presented for each sample (Figs. 3.14-3.15). One spectrum is of all ^{13}C resonances, the other is a non-quaternary suppression (NQS Delay or dipolar-dephasing) spectrum. The NQS spectrum shows resonances of ^{13}C carbons with weak coupling to ^{1}H. This is caused by either a long distance to the nearest ^{1}H (essentially, no direct ^{13}C-^{1}H bonding interactions, e.g. carbonyl, non-protonated aromatic, carboxyl groups); or by motion of ^{13}C-^{1}H bonds (the $-CH_3$ rotation averages dipolar decoupling and these ^{13}C signals are seen in NQS spectra, e.g. methoxyl groups).

In addition to the ^{13}C CP-MAS NMR spectra obtained for the galacturonan standards and separated zone tissue, other spectra of fruit tissues were also obtained. These are presented in Appendix A. They include a comparison of the unripe, ripening and ripe mesocarp (Appendix A, Fig. I); ripening unseparated zone and the tissues directly above and below it (Appendix A, Fig. II).

- **Galacturonan Standards (Fig. 3.14)**

The signal assignments for the C-6 carboxyl group in galacturonan (~177 and ~171 ppm) and the pectic methoxyl (~54 ppm) were confirmed with the high and low methylesterified lime pectins (Table 2.1, page 41) and polygalacturonic acid potassium salt (Section 2.13.1). Figure 3.14A-C shows the spectra of the galacturonan standards. In both high and low methylesterified pectin, the carboxyl of galacturonan is at ~171 ppm

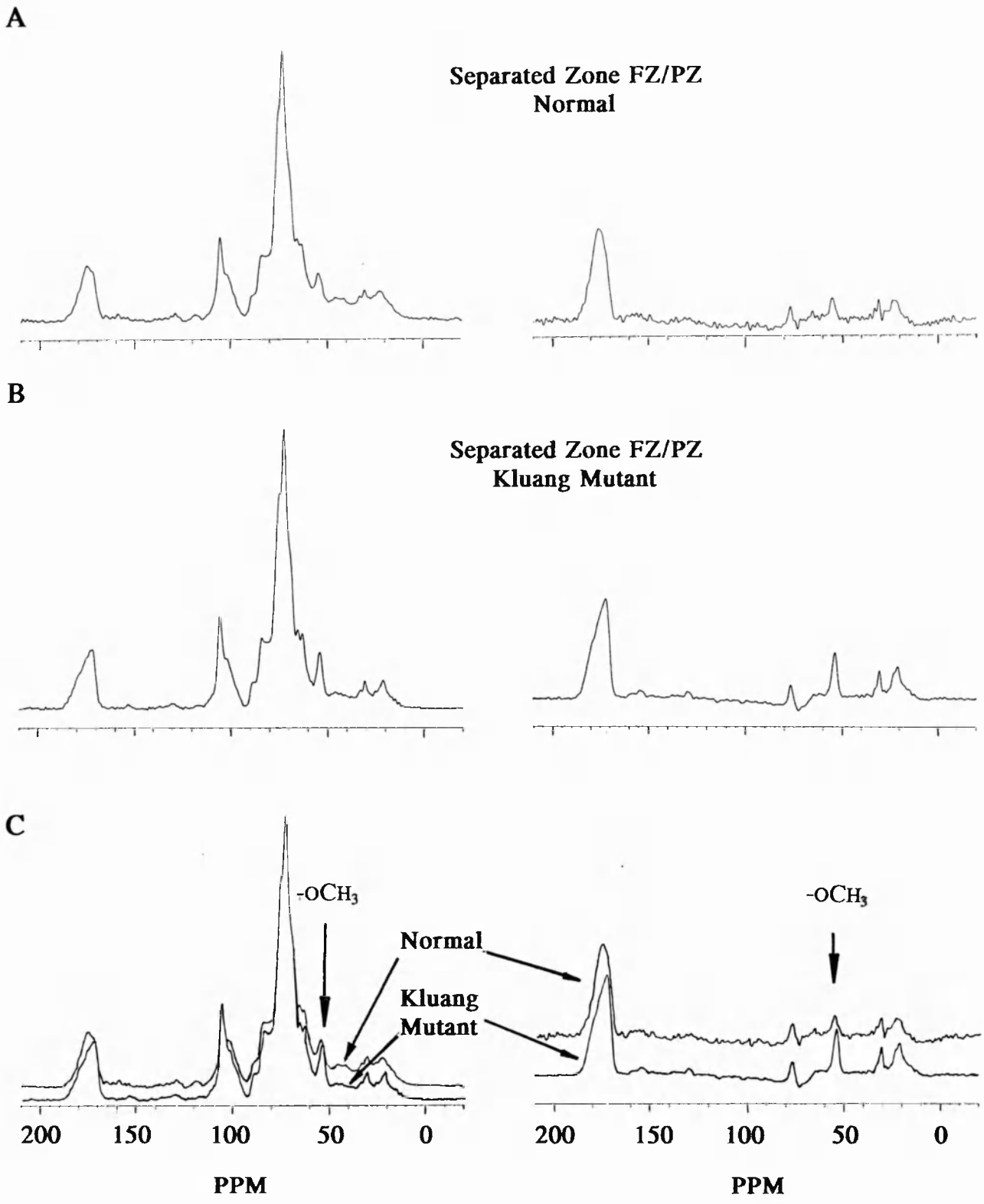


FIG. 3.15: ^{13}C CP-MAS NMR SPECTRA OF SEPARATED ZONE, FZ/PZ IN THE NORMAL AND KLUANG MUTANT FRUIT

The spectrum on the left is of all ^{13}C resonances and on the right, an NQS-Delay spectrum. In the Kluang mutant (B), the resonance for the methoxyl carbon at ~54 ppm is at least twice the intensity than that in the normal zone (A). The resonances at 171-177 ppm in the Kluang mutant (B) peak at ~171 ppm (methylesterified pectin) and this may indicate a different pectin structure to that in normal tissue. The two spectra are partially superimposed in (C) to emphasize these differences.

(Fig. 3.14A, B). The signal for the C-6 of galacturonan in polygalacturonic acid potassium salt is shifted downfield to ~177 ppm relative to methylesterified pectin at ~171 ppm (Fig. 3.14C). In addition, the low methylesterified pectin has a less intense signal for the methoxyl carbon at ~54 ppm than the high methylesterified pectin (Fig. 3.14A, B). Polygalacturonic acid has no signal at all for the methoxyl group at ~54 ppm. Resonances from all other carbons in galacturonan (C-1 to C-5) are seen between 60-110 ppm.

- **Comparison of Normal and Kluang Mutant Separated Zone Tissue (Fig. 3.15)**

Ripe Kluang mutant fruit separate very slowly and only after the spikelets are harvested, whereas the ripe normal fruit separate within 24h. Therefore, the slower separating ripening normal fruit (124 daa) which separated 72h after arrival in the laboratory were compared with ripe fruit of the Kluang mutant (201 daa) which also separated after 72h.

Figure 3.15 compares the spectra of normal separated FZ/PZ (A) with the Kluang mutant separated FZ/PZ (B) and these two spectra have been partially superimposed (C). The peak heights of all the resonances are very similar **except** for the peak at ~54 ppm for the methoxyl carbon, which is at least twice the intensity in the Kluang mutant. Since resonances from aromatics (lignin) also are absent (115-165 ppm) in the separated zone of the Kluang mutant, it would appear that there is more methylesterified pectin than in the normal separated zone.

In the absence of spectra from a preparation of purely unseparated cells, which cannot be obtained without inclusion of some mesocarp and pedicel tissue, a possibility exists that the walls of the zone cells of the Kluang mutant might be differentiated as more highly methylesterified than those of the normal fruit. Conversely, the pectin resonances observed in separated abscission zones of the Kluang mutant may instead reflect an altered pattern of pectin degradation during separation. For example, the role of pectin methylesterase may be different during cell separation. An indication that there is a different pectin composition after separation in the Kluang mutant was found when the polygalacturonase isoenzymes (pI 6.2 and 6.4) were extracted; there was selective retention of the pI 6.2 isoenzyme (which remained with the water-soluble pectins). This occurs only in the Kluang mutant fruit (Section 6.3.1).

3.6 SUMMARY AND DISCUSSION

Oil palm fruit do not soften appreciably during ripening, especially when compared with the avocado, another oil-bearing tropical fruit. Although the lipid content is high in the oil palm fruit (Table 3.1), there is a considerable amount of rigid fibre tissue (Figs. 3.9 and 3.10). The electron micrographs of oil-bearing mesocarp cells (Fig. 3.5A), showing the structural integrity of the cell wall and middle lamella, contrast with those of separated abscission zone cells (Fig. 3.8). This may reflect differences in the activity of certain enzymes like cellulase (see Table 5.1, page 117) and polygalacturonase (Chapter 6).

The higher carotenoid values obtained for the ripe non-abscinding Kluang mutant fruit (~400 μg g Fw^{-1}) compared with normal ripe fruit (~260 μg g Fw^{-1}) suggest that carotene biosynthesis continues even as the fruit become over-ripe (Fig. 3.4). Recently, attention has focused on the high carotenoid content in red palm oil for nutritional and health reasons (Vitamin A deficiency, cancer prevention). Crude palm oil is nature's richest source of carotenoids (up to 30 times greater than in carrots) but the regulation of carotene biosynthesis in the oil palm has not been explored. Thus, the non-abscinding mutant may have considerable economic potential and value in relation to carotenoid research.

Some degenerative processes may well occur as the mutant fruit become over-ripe (Fig. 3.2 & 3.3). Perhaps membrane disintegration in the oil-bearing cells initiates the subsequent cytoplasmic changes. It is not known if the TAG in the mutant is then hydrolysed by the lipase or if FFA content is higher than in normal fruit. This has important commercial implications and should be investigated on the plantation using freshly harvested fruit. As soon as samples are collected they need to be frozen immediately in liquid nitrogen to ensure accurate determination of the FFA content.

The non-abscinding behaviour of the Kluang mutant palm is not due to a difference in the anatomy of the abscission zone. The phloroglucinol/HCl staining of vascular connections did not reveal a difference between the normal and mutant fruit. After separation, the ^{13}C NMR spectra of normal and mutant zone tissue were very similar, except for the methoxyl peak (~54 ppm). This suggests that there may be a difference in

FIG. 3.16: LONGITUDINAL SECTION OF A FRUIT SEPARATED AT POSITION 1 ONLY

Complete separation has occurred at Position 1 but not at Position 2/3. The "translucency" does not extend to below the tepal bases (Position 4/5).

the middle lamella dissolution and/or cell wall disassembly in the mutant. However, this was a preliminary analysis to determine if there were any differences between the fruit tissues observable by ^{13}C CP-MAS NMR spectroscopy (the mesocarp, pedicel and unseparated zone tissues are shown in Appendix A, page 222). Further analyses of post-harvest separated zone samples of the Kluang mutant and of ripe fruit of other normal clones (including fruit of F1 seedling crosses) is planned. The ^{13}C CP-MAS NMR spectroscopic analyses showed that the separated zone spectrum was different from those of the mesocarp and pedicel (compare Fig. 3.15A with Fig. II A,B & C, page 224A). This confirmed the homogeneity of the tissue samples collected (for example, separated zone did not contain mesocarp lipid).

Both the Ni^{2+}/Na_2S staining and ^{13}C NMR analysis indicate that the abscission zone is pectin rich, especially in polygalacturonate (Figs 3.12 and 3.15A). In addition, when extracts of separated zone were dialysed at 4°C, pectin gel formation occurred (Section 2.4). The uronic acid analysis also indicates that pectin is present in only slightly lower amounts above and below the zone (Table 3.6). A study of the cell walls in tissues of monocotyledons (leaf, stem) reports a low pectin content in the grasses, cereals, sedges, rushes and woodrushes, with values of $\leq$50mg g^{-1} (Jarvis *et al*, 1988). These authors also estimated uronic acid in the pericarp of two monocotyledonous fruits, the date and banana, which had values of 199 and 208mg g^{-1} respectively. This is very close to the value obtained for oil palm fruit mesocarp in this study (209mg g^{-1}). The cell walls of dicotyledonous fruits have been reported with the following uronic acid contents: ripe tomato, 185mg g^{-1} (Koch & Nevins, 1989); ripe pear, 137mg g^{-1}; ripe kiwifruit, 33mg g^{-1} (Ahmed & Labavitch, 1977). In comparison with these fruit, the uronic acid content of ~340mg g^{-1} in the separated abscission zone confirms it has a very high pectin content.

Figures 2.1, 3.6B, 3.12A, 3.13B and 3.16 all show that the translucent zone at Position 1 extends only to just below the RA. Longitudinal sections of fruit just starting to separate show that Position 4/5 does not usually appear translucent (Fig. 3.16). However, when unripe/ripening unseparated fruit are held in tubes with their pedicel fibres in water for 48h (as shown in Fig. 3.13A) an extremely narrow band of translucency is observed below the base of the tepals. This suggests that pectin gelatinisation and hydrolysis also occurs when the tepals separate from their pedicel attachment. Whilst the

uronic acid analyses show that pectin is indeed present in pedicel tissue, the pectin composition and/or cell wall structure may be different in Position 4/5 from that in Position 1. Even pectin gel formation *in vitro* is highly complex. For example, in general, HM pectin (50-80%) forms a gel if the pH is below 3.6 and sugar is present, whereas in LM pectin (25-50%) a gel is formed in the presence of Ca^{2+} which acts as a bridge between pairs of carboxyl groups (Thakur *et al*, 1997). The delay in separation when spikelets were treated with additional calcium (Table 3.5) may indicate that a loss of Ca^{2+} from the oil palm zone is an accompaniment to abscission as shown by several authors (Stösser *et al*, 1969, for the fruit of the sour cherry; Osborne, 1989 and references therein). We shall be exploring this further by X-ray and PIXE (proton-induced X-ray emission) analysis of sectioned zone material.

The progression of abscission with resultant free carboxyl groups in pectin may be responsible, in part, for the increase in acidity in the zone which frequently has been observed (Osborne, 1989). We have measured low pHs of 3.0-4.0 (with wetted narrow-range pH paper) in the freshly separated FZ and PZ. Possibly some PME activity and/or the disruption of Ca^{2+} bridges in the pectin, along with the activity of endo- and exo-polygalacturonases induced at abscission (Chapter 6) all contribute to this low pH.

In conclusion, the abscission zone (Position 1) of oil palm fruit is pectin rich becoming translucent just prior to separation. Pedicel tissue also contains pectin but the Position 4/5 separation site (between the tepal bases and pedicel) appears to be different from Position 1. The contribution of the various cell wall hydrolase and pectolytic activities in fruit and tepal abscission is discussed in Chapters 5-8.

CHAPTER 4

ETHYLENE PRODUCTION OF NORMAL AND NON-ABSCINDING FRUIT

Ethylene production in normal fruit of clones 926 and 271D, and the non-abscinding fruit of the DxP5 Kluang palm are presented in this chapter.

4.1 EXPERIMENTAL MANIPULATIONS AND THEIR EFFECT ON ETHYLENE PRODUCTION

Ethylene can be produced in response to external stresses, for example, damage or removal of a plant organ. Therefore, it was important to know that the ethylene produced by the oil palm fruit in the laboratory was not due to removal of the fruit from the spikelet (Fig. 2.9, page 71A). The ethylene production of the intact spikelet or the fruit only from a spikelet was measured in large Kilner jars (1500ml); individual fruit were enclosed singly in smaller glass vials/bottles (28ml or 60ml depending on the size of the fruit). Kilner jars, bottles or vials were all sealed with lids containing a Suba-Seal and ethylene production was determined as described in Section 2.10.

4.1.1 Effects of Handling Spikelets and Fruit

It was essential to determine whether the manipulations of fruit on arrival resulted in any increased in ethylene produced by spikelets or fruit. To obtain the ethylene production of individual fruit and assess the stage of fruit separation, non-separating fruit were stripped from the spikelet (Fig. 2.9). The spiny floral bract was always removed and the bracteoles and tepals, depending upon the experiment, were also detached. The pedicel fibres were cut to a length of about 5mm.

The ethylene production of intact spikelets was compared with the production of a similar number of individual fruits that were removed from spikelets (as shown in Fig. 2.9). Ripe spikelets (162 daa) and those just starting to ripen (129 daa) of clone 926 were used and ethylene production determined for a measured closure period (for the ripe 162 daa, 1h; ripening 129 daa, 3h) immediately on arrival in the laboratory. The following table shows that in ripening fruit (129 daa) ethylene production is very low for intact spikelets (below 1nl C_2H_4 g Fw^{-1} h^{-1}). The ethylene production was generally higher

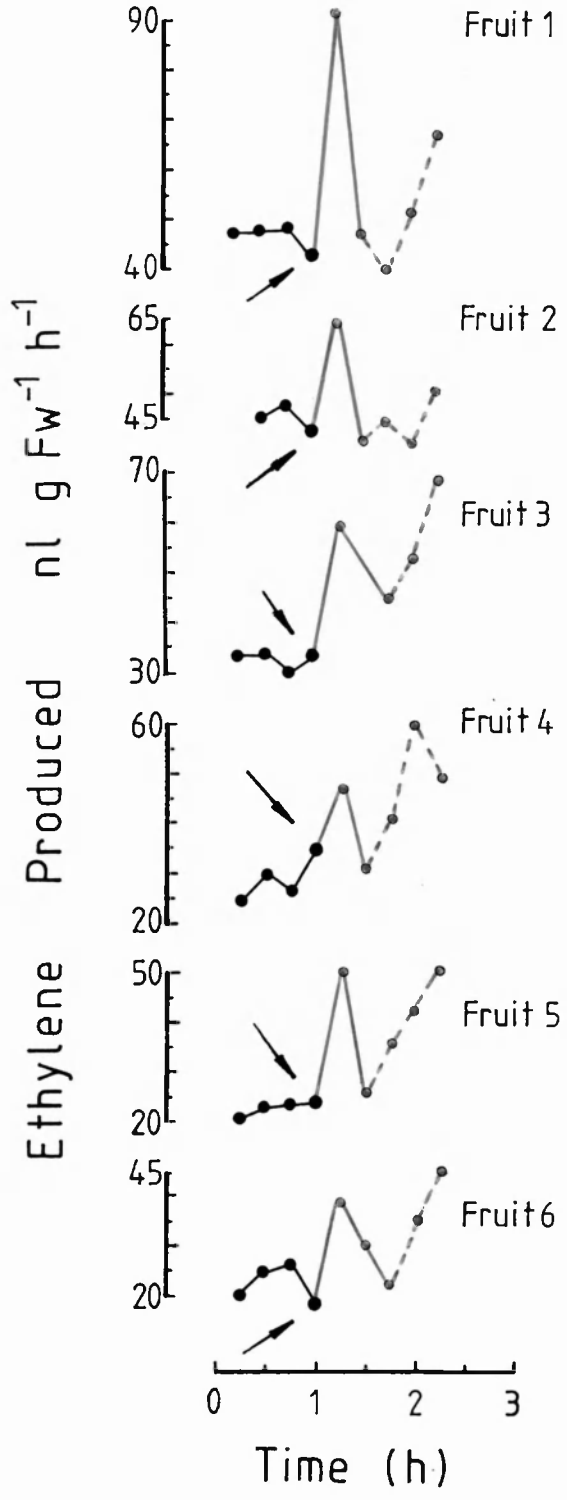


FIG. 4.1: ETHYLENE PRODUCTION IN RIPE FRUIT (CLONE 926) BEFORE AND AFTER WOUNDING

Ethylene production was monitored every 15min for 1h, fruit were then wounded by making five slices into the mesocarp and the ethylene production again monitored every 15min thereafter. The fruit were aerated after each 15min. The arrow indicates time of wounding.

in ripe spikelets (162 daa) on arrival (about 2-5nl C_2H_4 g Fw^{-1} h^{-1}) but some of these fruit had already started to separate and a spikelet with the highest ethylene production (5.4nl C_2H_4 g Fw^{-1} h^{-1}) shed four fruit. When the fruit are stripped from the spikelets, the ethylene production on stripping is not higher than that from intact spikelets (Table 4.1). These results indicate that the handling involved in the removal of fruit from the spikelet does not initiate a "wound" increase in the ethylene produced by the fruit.

RIPE, 162 daa	**nl C_2H_4 Produced g Fw^{-1} h^{-1}, on Arrival**
INTACT SPIKELET	
Number of fruit = 13	1.8
Number of fruit = 15	1.3
Number of fruit = 14	5.4
FRUIT REMOVED FROM SPIKELET	
Number of fruit = 14	0.0
Number of fruit = 14	3.3

RIPENING, 129 daa	**nl C_2H_4 Produced g Fw^{-1} h^{-1}, on Arrival**
INTACT SPIKELET	
Number of fruit = 18	0.1
Number of fruit = 16	0.3
Number of fruit = 16	0.0
FRUIT REMOVED FROM SPIKELET	
Number of fruit = 16	0.0
Number of fruit = 17	0.0

Table 4.1: Ethylene Produced by Fruit on the Intact Spikelet and All the Fruit Removed from a Spikelet (clone 926)

4.1.2 Wounding (by Slicing) the Fruit Mesocarp

For certain manipulations fruit were cut by longitudinal section (Fig. 2.9). Thus, it was necessary to know if there was a large ethylene increase in response to wounding and if this affected subsequent ethylene assays.

Ethylene production was monitored every 15min for 1 hour before wounding and and at 15min intervals after wounding in 6 ripe fruits (clone 926, 164 daa). The fruit were cut above the abscission zone, making 5 slices into the mesocarp. Figure 4.1 shows an immediate increase (within 15min) in ethylene evolution when fruits were cut in this way with a return to lower or pre-wounding levels within a further 15min (30min in

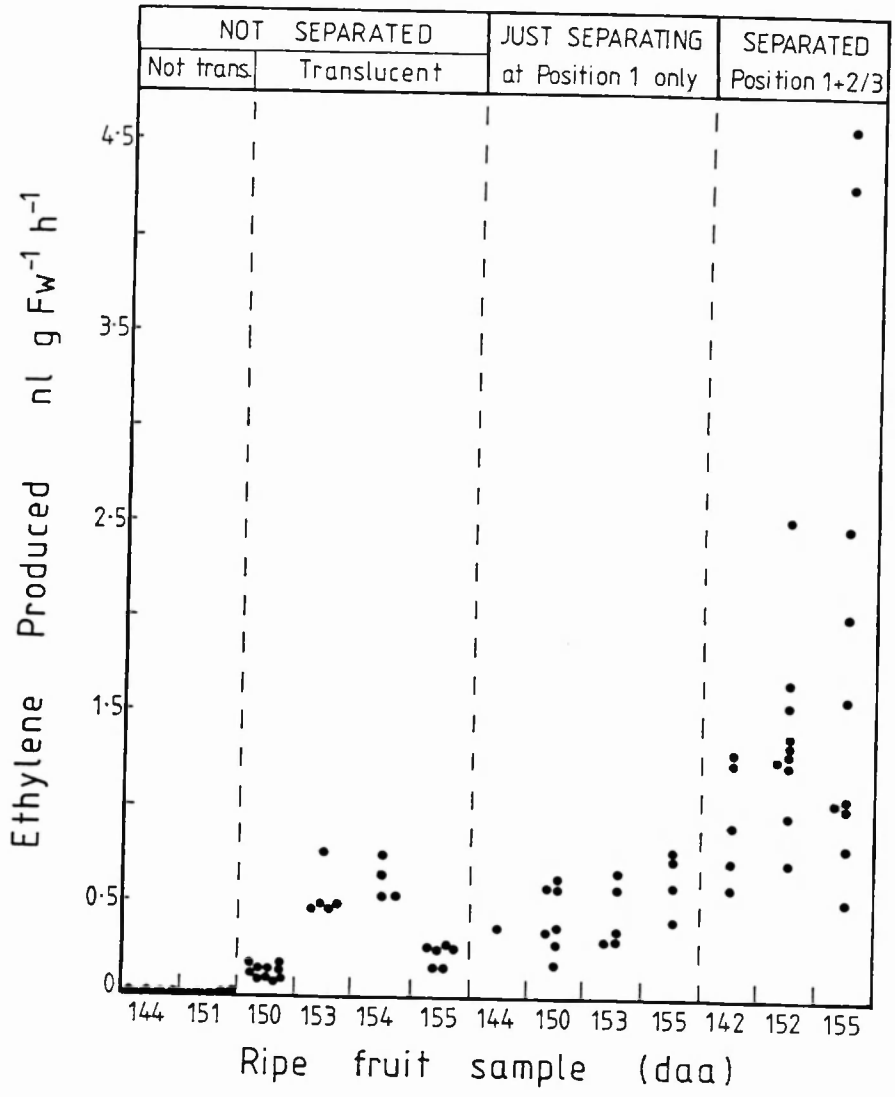


FIG. 4.2: ETHYLENE PRODUCTION IN NORMAL RIPE FRUIT (CLONE 271D) AND THE STAGE OF ABSCISSION

The ethylene production (30min closure period) was measured on arrival in the laboratory and then the fruit was assessed for the stage of separation at Position 1 by longitudinal section (Fig. 2.9, page 71A).

Fruit 6). However, the production by the fruit increased progressively thereafter. This suggests that ethylene soluble in the lipid was released on cutting, followed by the normal wound ethylene rise in response to damage.

4.2 ETHYLENE PRODUCTION AND FRUIT SEPARATION

4.2.1 Ethylene Production during the Different Separation Stages of Normal Fruit on Arrival

Figure 2.3 (page 46A, from Henderson & Osborne, 1994) shows that in clone 926 ripe fruit which had separated in transit had high ethylene production rates (up to 70nl C_2H_4 g Fw^{-1} h^{-1}) and ripe unseparated fruit had either zero or < 10nl C_2H_4 g Fw^{-1} h^{-1}. When fruit separated at Position 1, the ethylene production increased to 10-50nl C_2H_4 g Fw^{-1} h^{-1}. This pattern was investigated more closely in ripe fruit of clone 271D when it was apparent that a pre-separation stage (when the zone becomes translucent) was a prelude to the abscission event. **Immediately on arrival in the laboratory** the ethylene production of **ripe** unseparated and separated 1+2 fruit was measured (as described in Section 2.9) then directly after this, a longitudinal section of the abscission zone in fruit which had not separated revealed how far translucency had progressed at Position 1. In this way, the relationship between fruit abscission, translucency of the cells at the zone and ethylene production could be assessed. Table 4.2 and Fig. 4.2 show the increase in ethylene production as the abscission zone undergoes separation.

	nl C_2H_4 Produced g Fw^{-1} h^{-1}		
Abscission Zone Separation Stage, Normal Ripe Fruit on Arrival	**Range**	**Mean ± Standard Deviation**	**n**
Not translucent	<0.01-0.01	0.01 ± 0.00	20
Translucent	0.09-0.74	0.29 ± 0.22	25
Just starting to separate at Position 1	0.17-0.76	0.47 ± 0.18	16
Separated 1+2/3	0.38-4.59	1.53 ± 1.02	25

Table 4.2: Ethylene Produced by Ripe Normal Fruit (measured immediately on arrival, 0h) and Abscission Zone Separation Stage (unseparated fruit determined by longitudinal section, as shown in Fig. 2.9)

Figure 4.2 illustrates the spread of ethylene productions of individual fruit at each abscission stage. The change from non-translucent to translucent at Position 1 is very fast

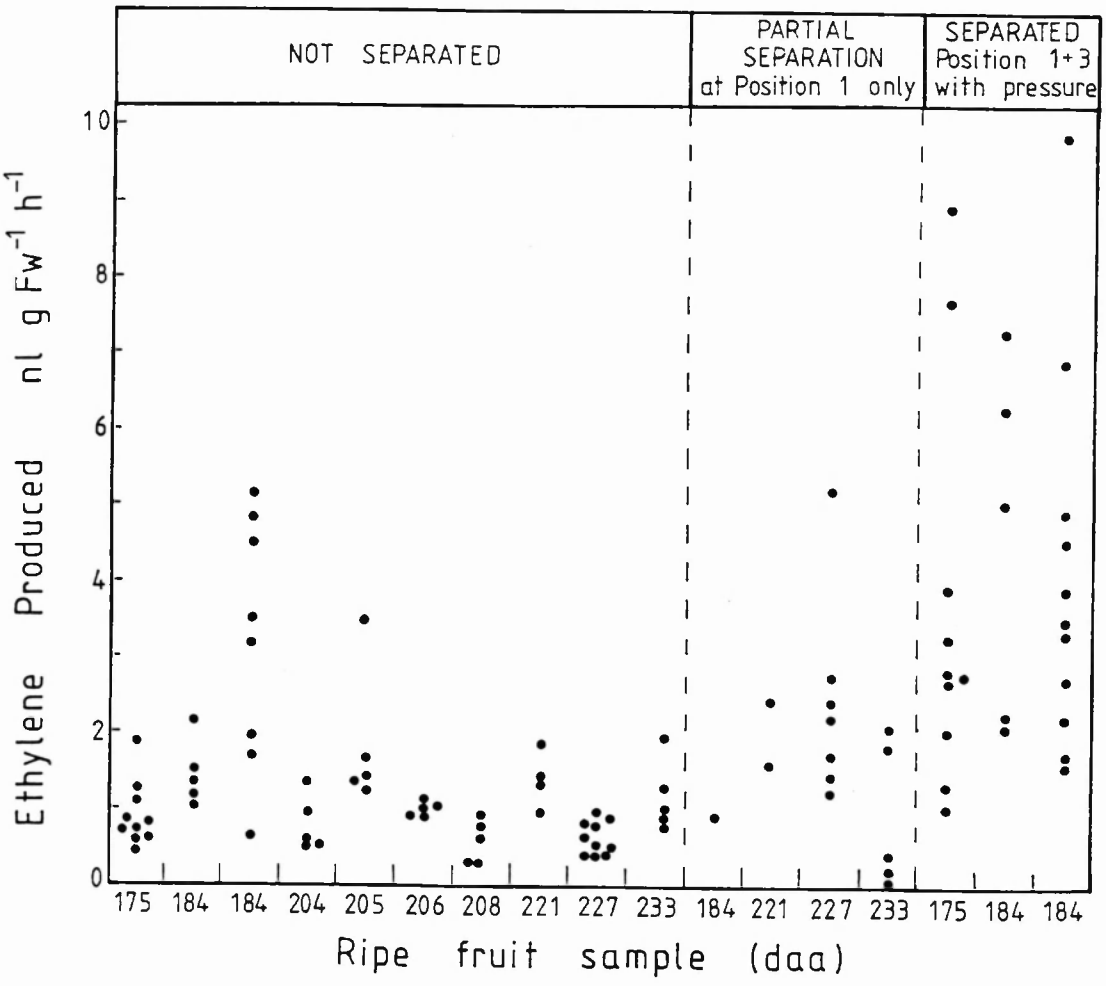


FIG. 4.3: ETHYLENE PRODUCTION IN KLUANG MUTANT RIPE FRUIT AND THE STAGE OF ABSCISSION

The ethylene production (30min closure period) was measured on arrival in the laboratory. The fruit was then immediately assessed for the stage of separation at Position 1 by longitudinal section (Fig. 2.9, page 71A).

(spanning less than 3-4h) but the differences in ethylene production are readily detected. These results confirm the similar pattern of ethylene production for clone 271D with that previously published for clone 926 (Fig. 2.3, page 46A). They show that the initiation of ethylene production in fruit closely parallels the sequence of events leading to fruit abscission.

4.2.2 Ethylene Production and Separation in Fruit from the Mutant Non-Abscinding Kluang Palm

These fruit do not separate in the field. Ripe, unripe and damaged fruit were therefore analysed for their ethylene production in the laboratory. The fruit were cut open by longitudinal section (Fig. 2.9, page 71A) and any separation of the zone was carefully recorded.

- **Ethylene Production and Separation in Ripe Mutant Fruit**

In contrast to the fruit of normal clones, the non-abscinding **ripe** fruit of the mutant Kluang palm were always producing ethylene on arrival (Fig. 4.3). Although **no** separation had been initiated at Position 1 and most fruit did not have translucent zones, all were producing between 0.3-5.0nl C_2H_4 g Fw^{-1} h^{-1} (compared with <0.01nl C_2H_4 g Fw^{-1} h^{-1} for normal ripe unseparated fruit and 0.09-0.74nl C_2H_4 g Fw^{-1} h^{-1} for normal fruit with translucent zones). The 15 fruit which showed separation at Position 1 or the 26 that were detached only with pressure, produced between 1-10nl C_2H_4 g Fw^{-1} h^{-1}. Figure 4.3 shows the spread of values for ethylene production in the individual fruit at 0h.

	nl C_2H_4 Produced g Fw^{-1} h^{-1}		
Abscission Zone Separation Stage, Kluang Mutant Ripe Fruit on Arrival (0h)	**Range**	**Mean ± Standard Deviation**	**n**
No separation at Position 1	0.34-5.13	1.30 ± 1.04	62
Partial separation at Position 1 only	0.12-5.29	1.81 ± 1.27	15
Separated 1+2/3 (with pressure)	1.08-9.96	4.07 ± 2.43	26

Table 4.3: **Ethylene Produced by Ripe Kluang Mutant Fruit (Measured Immediately on Arrival, 0h) and Abscission Zone Separation Stage (Unseparated Fruit Determined by Longitudinal Section, as shown in Fig. 2.9)**

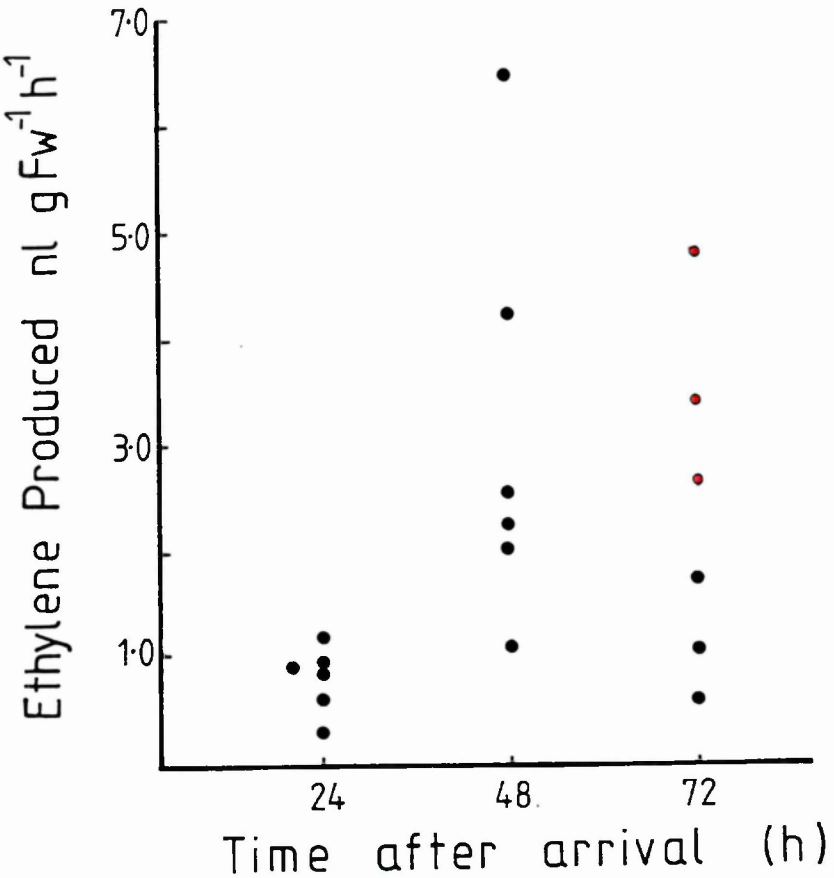


FIG. 4.4: THE POST-HARVEST ETHYLENE PRODUCTION OF KLUANG MUTANT FRUIT (194 DAA) AND FRUIT SEPARATION.

Ethylene produced by fruit increases with time after arrival in the laboratory but all fruit remain unseparated (•) at 48h. Position 1 separation is slow and by 72h after arrival (~96h since harvesting) only some fruit have partial separation (•).

On arrival in the laboratory (that is, 30h after harvesting), the ethylene production of the fully ripe undamaged mutant fruit was always higher than in normal fruit. Despite this, their **post-harvest separation** was considerably slower. Figure 4.4 shows the ethylene production of 194 daa Kluang mutant fruit. No separation occurred at all until 72h after arrival when only 3 of the 6 fruit analysed had some partial separation at Position 1 (checked by longitudinal section after the ethylene measurement). At 120h (5 days after arrival), 50% of the remaining fruit were still **not** separated at Position 1. This is in striking contrast with normal fruit abscission in which fully ripe fruit would all have separated in the laboratory by 24h. However, in the Kluang mutant, fruit separation did eventually take place in the laboratory, although it was delayed by several days relative to normal fruit and occurred at higher ethylene production rates (Table 4.4).

Fruit on one of the intact bunches of Kluang received (218 daa, 62 spikelets in all) were producing about 3 times more ethylene than other Kluang fruit analysed. Of the approximately 650-700 fruit on the bunch, only 2 fruit underwent complete unassisted abscission with separation at the fruit periphery (Position 1+2/3) and another 8 fruit could be removed (1+2/3) relatively easily. Twenty-three fruit were detached by pressure (1+3 separation), and the rest were very firmly attached. Samples of each of these classes of fruit were analysed for ethylene production before being cut open to observe the abscission zone.

	nl C_2H_4 Produced g Fw^{-1} h^{-1}		
Fruit Separation on Arrival (218 daa)	**Range**	**Mean ± Standard Deviation**	**n**
No separation at all	4-17 nl	10.8 ± 4.8	5
Partial separation at Position 1 only	10-28 nl	16.8 ± 7.4	5
1+3 separation with pressure	2-17 nl	9.9 ± 4.9	8
1+2/3 separated easily without pressure	5-24 nl	11.4 ± 5.8	10

Table 4.4: Separation Stage of Fruit in a High Ethylene Producing Kluang Mutant Bunch

Clearly, the high levels of ethylene synthesis by these Kluang mutant fruit was not sufficient to induce normal abscission. These very high ethylene productions by non-separating fruit were found consistently in all ripe bunches and spikelets received from this palm. After arrival in the laboratory the Kluang mutant abscission zone in unseparated fruit became translucent (see Section 3.2.2, page 83) and although separation of fruit did occur eventually in the laboratory, it was always delayed by several days relative to normal fruit. In general, it occurred at Position 1 only, or Position 1+4/5.

- **Ethylene Production and Separation in Damaged Mutant Fruit**

The very ripe and somewhat softer fruit of the Kluang mutant were easily damaged on the palm and in transit. The externally damaged fruit (Section 3.1.1, Figs. 3.1-3.3, pages 78-79) were examined for their ethylene production, since fungal contamination could give rise to the high rates observed. However, damaged fruit always produced less ethylene than intact fruit (Table 4.5 compare with Table 4.4 and Fig. 4.3).

	DAMAGED FRUIT (NOT SEPARATED) nl C_2H_4 Produced g Fw^{-1} h^{-1}		
	On Arrival	**After 48h**	**After 72h**
206 daa	0.06 ± 0.03 (n = 3)		0.70 ± 0.68 (n = 17)
218 daa	1.76 ± 1.59 (n = 4)		
233 daa	0.79 ± 0.73 (n = 5)	1.03 ± 0.51 (n = 5)	

Table 4.5: Ethylene Produced by Damaged Unseparated Ripe Fruit of the Kluang Mutant

On a bunch, damage is mainly to the terminal outer fruit of a spikelet and many of these do not separate at all in the laboratory. For example, 22 spikelets of 206 daa with damaged terminal fruit were placed in ethylene (10μl l^{-1}) for 72h. Separation was checked by longitudinal section (n=169):

Not Separated (all terminal fruit)	15.4%
Partial separation at Position 1 only (all terminal fruit)	4.1%
Separated **1+3** (all mid and lower fruit)	40.8%
Separated **1+4/5** (all mid and lower fruit)	39.7%

Terminal fruit can fail to separate even after 10 days in the laboratory although a few develop translucent zones. In normal clones in the field, terminal fruit are shed first, whereas in the laboratory the reverse occurs and lower fruit on the spikelet usually separate before the terminal fruit. The post-harvest distortion of the normal abscission sequence that results due to removal from the palm therefore operates also in the Kluang mutant.

- **Ethylene Production and Separation in Unripe to Ripening Mutant Fruit**

The fruit of one bunch of the Kluang mutant received (153 daa) appeared yellow with a greenish tinge at the stigma end of the fruit (that is, unripe). The ethylene production is shown in Table 4.6.

Kluang Mutant Fruit (153 daa)	nl C_2H_4 Produced g Fw^{-1} h^{-1}
Not Separated, on arrival	0.02 ± 0.01 (n = 6)
Not Separated, after 48h	0.04 ± 0.03 (n = 9)
Not Separated after 48h but had a translucent zone	0.64 (n = 1)

Table 4.6: Ethylene Produced by Unripe, Unseparated Kluang Mutant Fruit

After a further 24h (total 72h) in air, no fruit had separated (n=74) but there was enhanced separation in fruit held in ethylene (10μl l^{-1}, 72h) (Table 4.12, Section 4.3). However, **all the terminal fruit failed to separate**. Since the unripe 153 daa bunch did **not** have damaged terminal fruit, this low rate of abscission is not the result of external damage of the fruit or a lack of available ethylene.

Two further consignments of the non-abscinding Kluang mutant (150 daa and 156 daa) were received which both appeared pale yellow but with no obvious green tinge. Neither bunch showed abscission. The ethylene production of the 156 daa fruit on arrival was 0.03 nl C_2H_4 g Fw^{-1} h^{-1}.

When the ethylene production of a number of 150 daa intact spikelets of the Kluang mutant (2 in each of 3 Kilner jars) was measured after 72h in the laboratory it was 2.8 ± 1.0 nl C_2H_4 g Fw^{-1} h^{-1} (n = 3). On examination, none of the fruit showed any sign of translucency or separation at Position 1. This confirms that, although ethylene was being produced at 72h, it was not sufficient to induce any translucency or fruit abscission.

4.2.3 The Post-Harvest Ethylene Increase in Normal and Kluang Mutant Fruit

An enhanced ethylene production can be measured in any fruit soon after they are harvested (e.g. apple, melon). A similar post-harvest ethylene increase occurs in oil palm fruit, and the closer the fruit are to full-ripeness and natural abscission, the greater the ethylene increase.

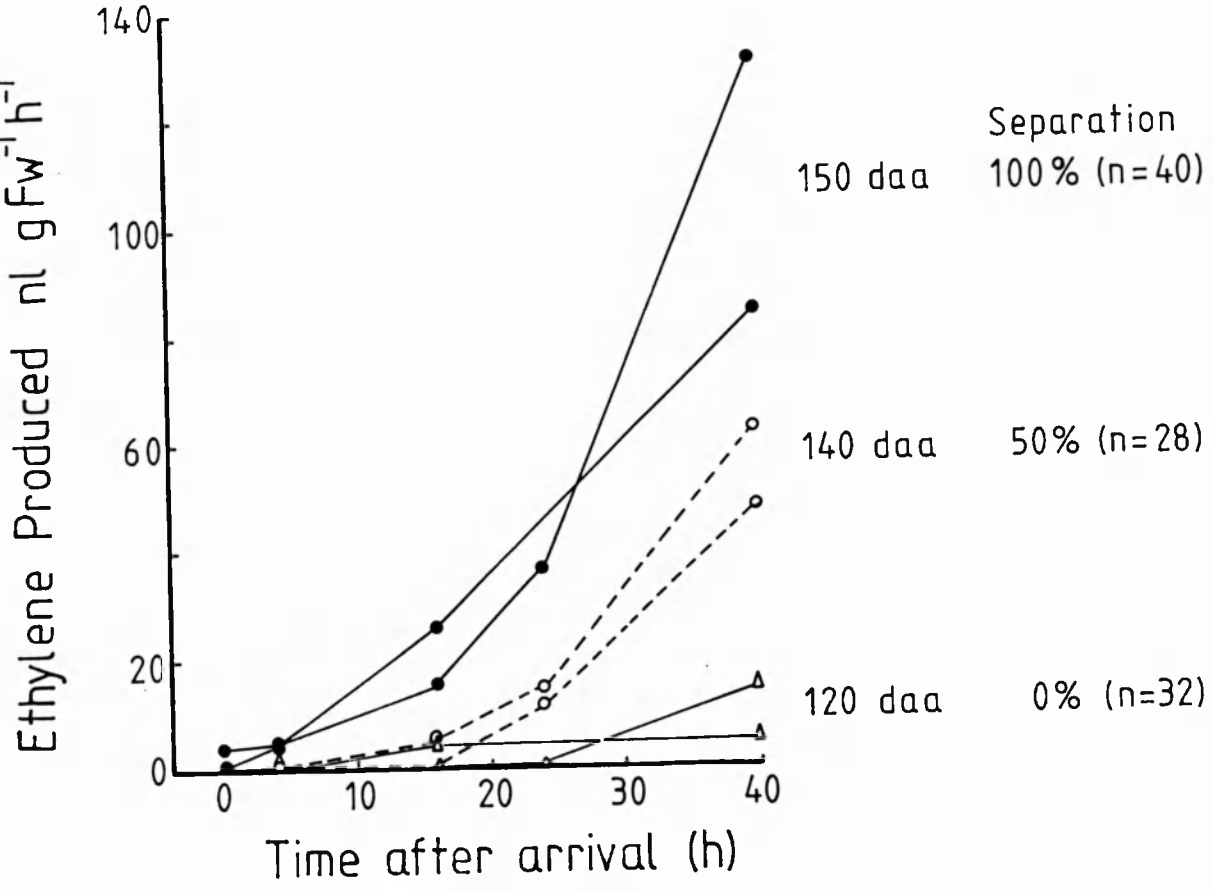


FIG. 4.5: THE POST-HARVEST ETHYLENE PRODUCTION IN SPIKELETS OF NORMAL FRUIT (CLONE 926).

Spikelets at the unripe (120 daa), ripening (140 daa), and fully ripe (150 daa) stages were measured for ethylene production after arrival in the laboratory and during the following 40h. Two spikelets of each age were monitored and each spikelet was held (90min closure period) in a 1.5 litre Kilner jar. After the final ethylene measurement, the spikelets were examined for fruit separation.

- **Unripe, Ripening and Ripe Spikelets (Clone 926)**

Immediately on arrival, spikelets of 120 daa, 140 daa, and 150 daa (2 spikelets of each age, 1 in each 1.5 litre Kilner jar) were measured for 40h after closure periods of 90min with aeration between measurements. Ripe spikelets start to produce high levels of ethylene after 15h increasing over the next 25h, whereas ripening and unripe spikelets produce lower levels starting later (25h and 40h respectively) (Fig. 4.5).

- **Ripe Fruit, either Abscinded in Transit or Close to Separation (Clone 926)**

Three ripe terminal fruit (154 daa) which abscinded in transit (Fig. 4.6A, page 107A) were examined for their post-abscission ethylene production (a closure period of 30-60min was used). Ethylene continued to rise in 2 fruit but in all 3, it started to decrease after 3.5h. Three unseparated ripe mid-fruit from the same spikelets which separated 9.5h after arrival in the laboratory were also followed for comparison (Fig. 4.6B, page 107A). Ethylene production increases from undetectable/very low levels and continues to increase after separation at 9.5h after arrival. This pattern was confirmed by following the ethylene production of ripe unseparated fruit (164 daa, n = 15) over 50h after arrival in the laboratory (Fig. 4.7, page 107A). In these fruit, ethylene production continues to increase for a short period only, after most fruit have separated but then gradually decreases as observed for the fruit shed in transit (Fig. 4.6A, page 107A).

- **Comparison of Terminal, Mid and Lower Fruit on the Spikelet (clone 926)**

The ethylene production of the terminal (outer), mid (central) and lower (inner) ripe fruit (Fig. 1.5, page 32A) on the spikelet was measured on arrival (0h) or at 3h (after a closure period of 30min). The fruit were then cut open to assess the stage of separation at the abscission zone and ethylene production was monitored again at 3h and 4h after cutting the fruit open. On arrival (0h), the terminal fruit (some of which had separated in transit and some had already separated at Position 1) were producing more ethylene than the mid or lower fruit on the spikelet. However, from 3h-10h there was no positional difference in the post-harvest ethylene production (endogenous or wound) by terminal, mid and lower fruit of the spikelet as shown in Table 4.7.

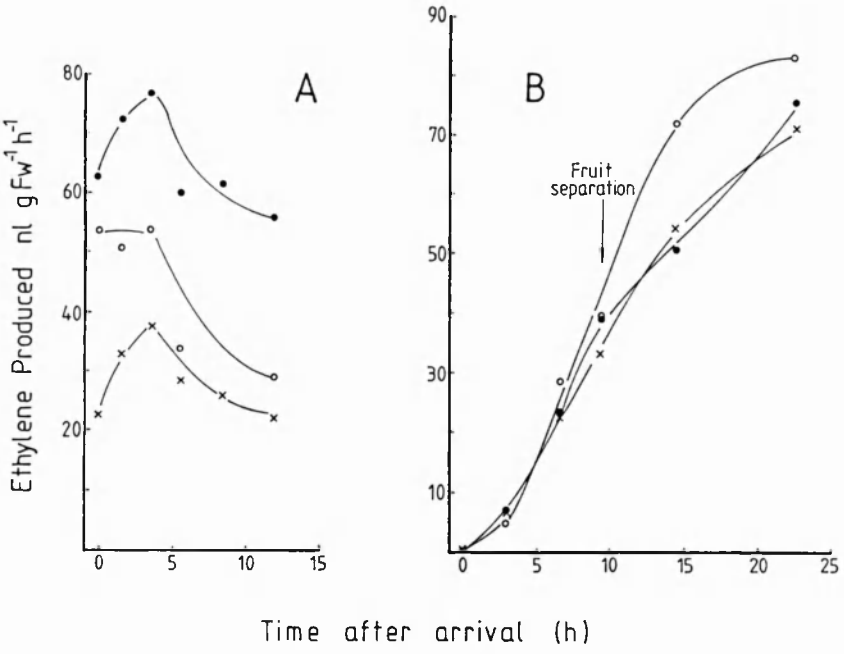


FIG. 4.6: THE ETHYLENE PRODUCTION OF RIPE FRUIT (CLONE 926, 154 DAA) SHED IN TRANSIT (A) AND THOSE WHICH SEPARATED 9.5H AFTER ARRIVAL (B).

Three fruit which shed in transit and three fruit which all shed at 9.5h were selected to show the post-abscission ethylene production (20-30min closure periods).

A. Three terminal fruit shed in transit; high ethylene production continues for 3.5h but then decreases.

B. Three fruit unseparated on arrival in the laboratory; initially ethylene production is not detected/very low; production rises and continues to increase as the fruit separates (9.5h after arrival).

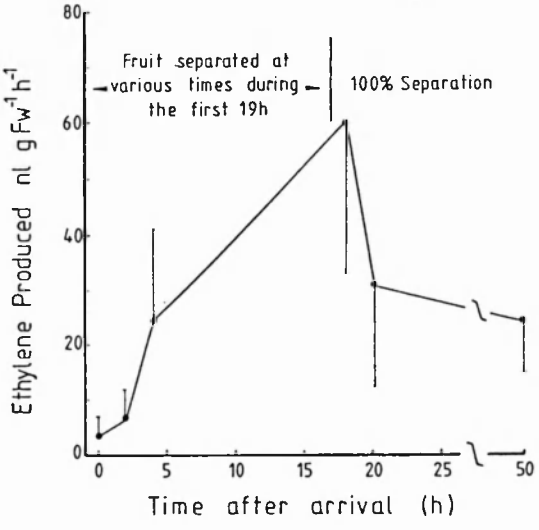


FIG. 4.7: THE POST-HARVEST ETHYLENE PRODUCTION IN RIPE FRUIT (CLONE 926, 164 DAA).

Individual fruit close to separation (n = 15) were measured for their ethylene production (60min closure periods) on arrival in the laboratory and at various times during the following 50h. All fruit had separated by 19h after arrival in the laboratory. The average production for the 15 fruit is calculated at each measurement (bar = s.d).

		nl C_2H_4 Produced g Fw^{-1} h^{-1}		
	Time after Arrival	TERMINAL	MID	LOWER
INTACT RIPE FRUIT (154 daa)	0h	0.9; 7.2 (n=2)	0.03 ± 0.06 (n=3)	0.4 ± 0.2 (n=3)
	3h	5.1 ± 1.4 (n=3)	3.3 ± 4.1 (n=3)	6.1 ± 1.9 (n=4)
INTACT FRUIT ABOVE CUT OPEN AT EITHER 0h OR 3h	6h	28.1 ± 6.1 (n=5)	28.8 ± 9.0 (n=6)	33.7 ± 11.2 (n=7)
	10h	40.0 ± 6.9 (n=5)	43.9 ± 12.2 (n=6)	42.2 ± 17.9 (n=7)

Table 4.7: Ethylene Produced by the Terminal, Mid and Lower Fruit on a Spikelet.

- **The Contribution to Ethylene Production by the Spikelet and Tepals (Clone 926)**

The ethylene production of intact spikelets and spikelets from which fruit were removed was measured starting 30h after arrival in the laboratory. The unseparated fruit of 140 daa spikelets were removed by cutting; fruit of 157 daa spikelets which had separated at Position 1 were easily detached by hand. The spikelets were enclosed in 1.5 litre Kilner jars for a closure period of 1h for spikelets with fruit and for 5h for those spikelets with fruit removed. Ethylene production was then determined. In neither ripening nor ripe samples was the contribution of the vegetative parts greater than 17% of the total post-harvest ethylene production (Table 4.8).

	nl C_2H_4 Produced g Fw^{-1} h^{-1}	
Clone 926	Fruit + Spikelet	Spikelet + Tepals Only
Ripening (140 daa)	102.6	11.6
	70.8	11.9
Ripe (157 daa)	49.5	6.1
	75.3	6.5

Table 4.8: Ethylene Produced by the Spikelet with Fruit and the Spikelet with Tepals alone

- **The Post-Harvest Ethylene Production in Ripe Fruit of Clone 271D**

Fruit of clone 271D showed a similar pattern of increased ethylene production, as separation occurred, to that measured in other clones. Measurements were taken only until

the time that the fruit were shed and, although ethylene production was lower in this clone than in clone 926, the increase was still observed (Table 4.9).

Ripe Fruit (155 daa)	nl C_2H_4 Produced g Fw^{-1} h^{-1}
Not Separated, on arrival (n = 6)	0.2 ± 0.1
Separated on arrival (n = 10)	2.0 ± 1.4
Separated 24h after arrival (n = 10)	6.1 ± 2.7

Table 4.9: The Post-Harvest Ethylene Increase in Ripe Normal Separated Fruit

A contributory factor in the post-harvest ethylene production may be the change in the physiological state of the fruit which, after harvesting, initiates separation processes resulting in the shedding of even unripe fruit. When the spikelets have been excised from the parent palm, the fruits are deprived of their normal supply of water and nutrients and, if the fruit continue to transpire, this would result in a weight loss. To ascertain this, the weight loss of ripe normal spikelets and spikelets without fruit was measured over a period of 3 days. These fruit (152 daa) were very close to complete separation and some had already separated in transit. Table 4.10 shows there is a 20% weight loss from spikelets with fruit in 3 days. The weight loss of the stem of the spikelet (no fruit) is 5.5%. Thus, the loss is greatest from the fruit, about 15% of their weight, and ~5% each day. The weight loss is linear over the 3 days.

Ripe Fruit Spikelets (152 daa)	Weight (g) ± Standard Deviation				Weight Loss in 72h ($\bar{x}$ ± sd)
	0h	24h	48h	72h	
Spikelets with fruit n = 10	25.6 ± 4.8	23.4 ± 4.7	22.4 ± 4.6	20.5 ± 4.8	5.1 ± 1.1 **(20.3%)**
Stem/tepals of spikelet only (no fruit) n = 20	11.0 ± 1.3	10.7 ± 1.3	10.6 ± 1.3	10.4 ± 1.3	0.6 ± 0.1 **(5.5%)**

Table 4.10: The Post-Harvest (0h to 72h) Decrease in Weight of Fruit Spikelets

Water deficit stress has been reported to promote ethylene synthesis in detached plant parts (Morgan *et al*, 1990). The weight loss (5% each 24h) observed in harvested spikelets with fruit may contribute to the induction of ethylene synthesis, especially in unripe/ripening fruit, which then results in fruit abscission.

Bafor & Osagie (1986) noted that normal fruit which became over-ripe (182 daa) before harvesting (in Nigeria) appeared shrivelled and desiccated. It would seem that after separation at the abscission zone, the continual loss of water from the fruit leads to dehydration. Therefore, since the non-abscinding mutant fruit do not appear shrivelled or desiccated whilst still on the palm, this suggests there is complete attachment at the abscission zone and that either no separation or very little separation occurs at Position 1.

- **The Post-Harvest Ethylene Increase in the Kluang Mutant**

Ethylene levels of ripe unseparated Kluang mutant fruit were greater than in the normal unseparated fruit. However, when mutant fruit did separate in the laboratory, although ethylene production increased, it did not reach the high levels observed in clone 926 (during the same time period, ~48h).

	nl C_2H_4 Produced g Fw^{-1} h^{-1}	
Kluang Mutant Fruit	**Not Separated, 0h**	**Separated, 48h**
RIPENING 153 daa	0.03 ± 0.02 (n = 6)	2.28 ± 1.32 (n = 8) Position 1 only
RIPE 221 daa	1.68 ± 0.49 (n = 6)	3.41 ± 1.09 (n = 7) Position 1 only
RIPE 233 daa	1.22 ± 0.46 (n = 5)	5.63 ± 2.26 (n = 5) 1+2/3 and 1+4/5

Table 4.11: The Post-Harvest Ethylene Produced by Kluang Mutant Fruit

4.3 THE EFFECT OF APPLIED ETHYLENE IN THE NORMAL AND KLUANG MUTANT FRUIT

Previous published work (see Fig. 3 in Henderson & Osborne, 1994; Appendix E) showed that in normal fruit applied ethylene enhanced separation at Position 1. Unripe (118 daa), ripening (128 daa, 138 daa) and near-ripe (148 daa) fruit of the normal clone 926 were placed in glass tanks (12 litres) in either ethylene (5μl l^{-1}) or in air + mercuric perchlorate (to absorb ethylene). Cell separation at Position 1 occurred earlier in fruit exposed to ethylene than in those fruit from which ethylene was depleted from the ambient

air by the mercuric perchlorate. The acceleration due to the additional ethylene was less in the fruit nearing full ripeness (148 daa).

Since the ripe Kluang mutant fruit were already producing ethylene on arrival, unripe to ripening fruit (which had very low ethylene production) were used to examine whether applied ethylene would accelerate separation at Position 1 (Table 4.12). Spikelets were placed in the glass tanks (12 litres) in either ethylene (10μl l^{-1}) or in air + MP for 72h (with aeration every 24h).

Kluang Mutant (153 daa)	**C_2H_4 (10μl l^{-1})**	**Air + MP**
	(n = 90)	(n = 74)
No separation	20.0% (all terminal fruit)	100%
Separated 1+3	15.5% (mid and lower fruit)	
Separated 1+3 with pressure	25.6% (mid and lower fruit)	
Separated 1+4/5	38.9% (mid and lower fruit)	

Table 4.12: The Effect of Applied Ethylene (10μl l^{-1}, for 72h with aeration every 24h) on Separation in Kluang Mutant Fruit

The unseparated fruit in air showed no separation at all at Position 1 when examined by longitudinal section. However, 66% of the unseparated terminal fruit in ethylene showed some partial separation at Position 1 when checked by longitudinal section (12 of the 18 terminal fruit). The majority of the fruit in air eventually separated by 168h after arrival.

The effect of ACC or ethylene was examined in another sample of ripening Kluang mutant fruit (156 daa). Fruit were incubated for 24h with their pedicel fibres in a solution of ACC (1mM) or water; ethylene (10μl l^{-1}) or air + mercuric perchlorate (MP). A longitudinal section of the fruit showed that both ACC and ethylene enhanced separation at Position 1 only (Table 4.13).

	% SEPARATION AT POSITION 1	
Kluang Mutant (156 daa)	**ACC (1mM)**	**H_2O**
Fruit number = 5	80%	0%
	C_2H_4 (10μl l^{-1})	**Air + MP**
Fruit number = 11	100%	0%

Table 4.13: The Effect of ACC or Ethylene (24h) on Separation in Kluang Mutant Fruit

Although the separation of mutant fruit was enhanced with ACC or ethylene treatments, to remove the fruit still required some pressure. This indicated that their separation was not identical to that in normal fruit, and Position 1 only was involved.

4.4 SUMMARY AND DISCUSSION

There is no measurable ethylene production during ripening (~120-150 daa). It is detected only when a fruit is fully ripe and very close to abscission. Although the fruit of clone 926 produced much higher levels of ethylene than those of clone 271D (Fig. 4.7, compared with Fig. 4.2), both clones have undetectable (or extremely low) levels of ethylene during the ripening period. Fruit producing a low level of ethylene, when examined by longitudinal section, always show either a translucent abscission zone or partial separation at Position 1. Ethylene production then rapidly increases up to complete separation of the zone (1+2, 1+2/3, 1+3) and for some time thereafter (possibly a few days). On the plantation some abscinded fruit drop from the spikelets to the ground (a sign to harvest the large fruit spike, Fig.1.4, page 30A) whilst others, although separated, remain loose in the socket. The increased ethylene production by these loose fruit no doubt would then affect the surrounding ripe fruit, accelerating their abscission. This is a possible explanation for the observation by plantation staff that once separation starts, it proceeds very rapidly and is the reason why the bunch is harvested as soon as a few fruit have been shed.

Ethylene is synthesized when a solution of ACC (0.1-1mM) is applied to unripe or ripening mesocarp tissue (Henderson & Osborne, 1994). Therefore, ACC oxidase is present in the mesocarp throughout development, although no detectable ethylene is produced by the fruit until abscission and possibly at this time ACC synthase is induced.

A

B

FIG. 4.8: THE APPEARANCE OF RIPENING AND RIPE FRUIT 10 DAYS AFTER HARVESTING

A. Ripening fruit (120 daa). No post-harvest carotene synthesis is observed.

B. Ripe fruit.

However, **detached** unripe and ripening oil palm fruit **do not** ripen, even when ethylene is applied. No post-harvest carotene accumulation is evident, and if TAG is present it undergoes rapid hydrolysis by a lipase (Section 1.3.1, page 30). The fruit do, however, begin to produce a low post-harvest level of ethylene but this results only in abscission from the spikelet. The post-harvest changes in oil palm, despite increased ethylene production by the fruit, are degenerative as shown in Fig. 4.8. The fruit "brown" and dehydrate, with the signs of deterioration, starting at the fruit base, becoming visible within a few days (Fig. 4.8 and Section 4.2.3). It appears, therefore, that carotene biosynthesis in the oil palm mesocarp is not linked to ethylene production, as is lycopene synthesis in the harvested tomato. Thus, in oil palm fruit, ethylene production is necessary for normal fruit abscission but increased levels are not required in fruit ripening.

In the Kluang mutant, carotene and triacylglycerols are synthesized and ripening appears to proceed as for normal fruit. When fruit are ripe, ethylene is produced but this does **not** result in abscission. The lesion of this mutant is not in its ethylene biosynthesis and probably not in the perception of ethylene since, once harvested, the mutant fruit do eventually separate with the induction of cell wall hydrolases (cellulase, polygalacturonase, laminarinase etc.) which are known to be regulated by ethylene in other abscission systems (bean pulvinus, tomato flower, avocado fruit).

An increased ethylene production at fruit abscission is also observed in fruit which produce relatively high levels during fruit ripening like the melon (a "climacteric" fruit). Dunlap *et al* (1996) report the onset of greatly increased ethylene production (more than 5-fold) at the same time that the initial stages of abscission occur (identified by partial separation of tissue in the fruit abscission zone). This is further supported by the results of Aggelis *et al* (1997), who have shown that, in two melon varieties (Alpha and Sirio), *MEL*1 mRNA (ACC oxidase) is not detected when the fruit changes colour from green to orange at about 30 daa, but significant amounts are produced at 40 daa when fruit usually abscind from the vine. Whilst Dunlap *et al* (1996) reported ethylene levels of about 5-10 nl g Fw^{-1} h^{-1} during melon development, the levels in ripening oil palm are undetectable. Ripening occurs over a much longer period than abscission. In the oil palm, fruit ripen in 30 days, whereas the fruit abscind in as many hours. The surge of ethylene production at abscission appears to reflect this difference in both the melon and oil palm, although the levels of ethylene during ripening in the two fruit are very different.

The melon can be harvested before full fruit maturity but this is not possible for oil palm fruit. The post-harvest behaviour of "mature-green" oil palm fruit contrasts with many other fruit where detachment often hastens the onset of ripening. For example, apples are pre-disposed to ripen when harvested and, shortly after detachment, a large increase in internal ethylene concentration occurs whilst the internal level of ethylene in attached fruits remains unchanged for many more days (McGlasson *et al*, 1978). Thus, the oil palm appears to fit the "non-climacteric" pattern of ripening. Most fruits of this type (e.g. the orange) need to complete ripening before harvest because desirable post-harvest changes are usually limited (McGlasson *et al*, 1978). The biosynthesis of fatty acids, TAG and carotene requires ATP, NADH and acetyl CoA. Detached mature-green tomato will start to synthesise ethylene and begin their colour change, progressing to deep red, due to the synthesis of lycopene (Salveit, 1993) but the regulation of carotenoid (and TAG) synthesis in the oil palm must be quite different. Our electron micrographs (Fig. 3.5) do not indicate that the oil palm mesocarp contains large reserves of carbohydrate (starch grains) for use in post-harvest syntheses.

A comparison of the ethylene production in the fruit of the oil palm and the date palm (*Phoenix dactylifera*) reveals an interesting difference. As shown in this chapter, ethylene production in the oil palm occurs at full fruit ripeness, when tricacylglycerol biosynthesis has reached its maximum with resultant fruit abscission and the subsequent hydrolysis of TAG. However, in the date fruit, although ethylene is not detected early in development, it begins to increase and peaks during the phase when the fruit becomes physiologically mature (Khalal stage) at which time it attains its maximum sucrose content (~80%). Abbas and Ibrahim (1996) report that ethylene production occurs during the Khalal stage (a period of about 25 days) and production peaks at 3.6nl C_2H_4 g Fw^{-1} h^{-1} (measured in fruit collected directly from the palm). The ethylene production then returns to undetectable levels as the dates start to dehydrate, carotenoids are lost and the fruit colour changes from red/yellow to brown, until finally the dates are "cured". Fruit do **not** abscind from the bunch and are harvested by hand (Rygg, 1975; Nixon & Carpenter, 1978). Our own experiments with some harvested Asseel dates seem to support the conclusion that "ripening" changes in the date involve dehydration of the "self-preserved" fruit. It would be interesting to know if some partial abscission occurs at the pedicel fruit junction at the peak of ethylene production. This possibility is suggested by the

subsequent changes in the fruit all of which are characteristic of senescence: dehydration, no further accumulation of sucrose, loss of carotenoids, and browning of the fruit.

In summary, for oil palm fruit, ethylene production is not detected during the ripening period of ~30 days (but may be present in the tissues at **extremely low levels**). A signal at full fruit ripeness results in a huge increase in ethylene production within only a few hours, followed by an equally fast fruit abscission. The oil palm fruit illustrates that ethylene levels required to cause abscission are not those related to fruit ripening and oil palm cannot be classed as a fruit with an ethylene "climacteric" (Somasundram *et al*, 1994). Therefore, investigations of ethylene production during ripening should also be correlated with the abscission behaviour of a fruit.

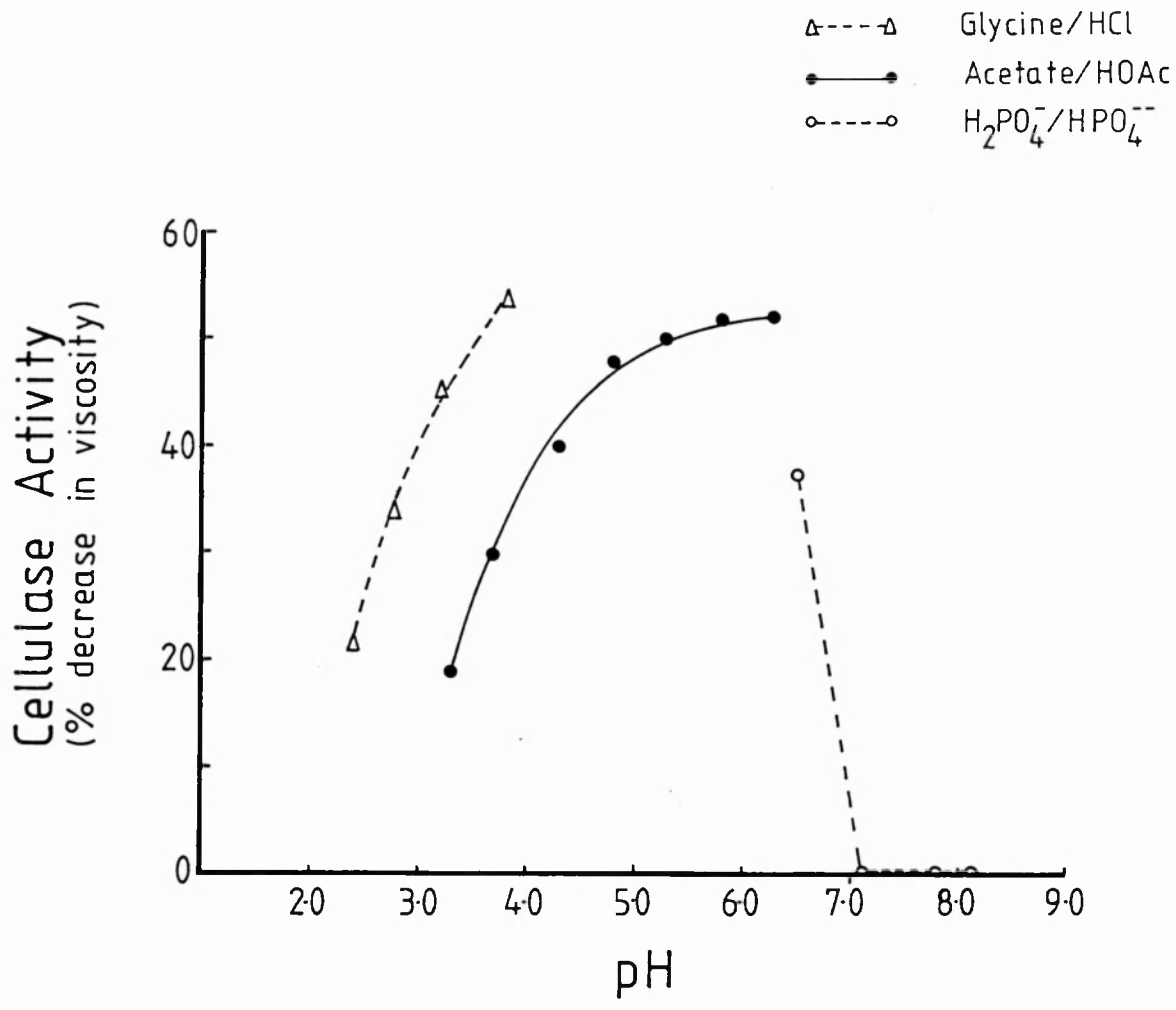


FIG. 5.1: THE pH DEPENDENT ACTIVITY OF CELLULASE IN MESOCARP TISSUE

The cellulase activity (% decrease in viscosity in 1h for 50μl enzyme extract) was examined in three different buffers (100mM glycine, 100mM acetate and 100mM phosphate) between pH 2.4 and 8.1.

CHAPTER 5

ENDO-ß-1,4-GLUCANHYDROLASE (CELLULASE) ACTIVITY

5.1 CELLULASE ACTIVITY IN RIPENING AND RIPE NORMAL FRUIT

Endo-ß-1,4-glucanhydrolase, or cellulase, activity was assayed with carboxylmethyl cellulose substrate (CMC) (Section 2.6.3). Activity was detected in extracts by a decrease in the viscosity of a solution of CMC (~1.5%) when incubated (30°C) between 1-4h. The cyanoacetamide assay for reducing groups was not as sensitive as the viscosity assay, and reducing groups were only just detectable after a long incubation (up to 24h). Hatfield & Nevins (1986) found that avocado cellulase rapidly reduced the viscosity of a CMC solution, whereas the increase in reducing groups was linear and relatively slow. Reducing groups increased even after the loss in viscosity was complete, and this is characteristic of the hydrolysis of internal linkages showing that the enzymes are endo-hydrolases.

5.1.1 Cellulase Activity in the Mesocarp

Cellulase activity was not detected in unripe (as young as 57 daa) and ripening mesocarp but it was shown to be present first in mesocarp of ripe fruit just prior to separation. An enzyme extract then reduced the viscosity of a solution of CMC (as described in Section 2.6.1) by approximately 50% in 1h.

The pH dependency of the enzyme was examined from pH 2.4-3.8 in 100mM glycine/HCl buffer, from pH 3.3-6.3 in 100mM acetate buffer and pH 6.5-8.1 in 100mM phosphate buffer and is shown in Fig. 5.1. (The buffering capacity of glycine/HCl is between pH 2.2-3.6; acetate, pH 3.7-5.6; and phosphate, pH 5.8-8.1; Data for Biochemical Research, 1969.) The mesocarp was extracted in deionised water containing 1M NaCl and protease inhibitors. The pH of the buffer in the assay then remained unchanged when enzyme extract was added.

In glycine/HCl buffer activity was relatively high even at pH 2.4 and between pH 3.2-3.8 cellulase activity was twice that obtained in acetate buffer at the same pH. The highest activity was obtained in acetate buffer pH 5.3-6.3 and glycine/HCl pH 3.8, and

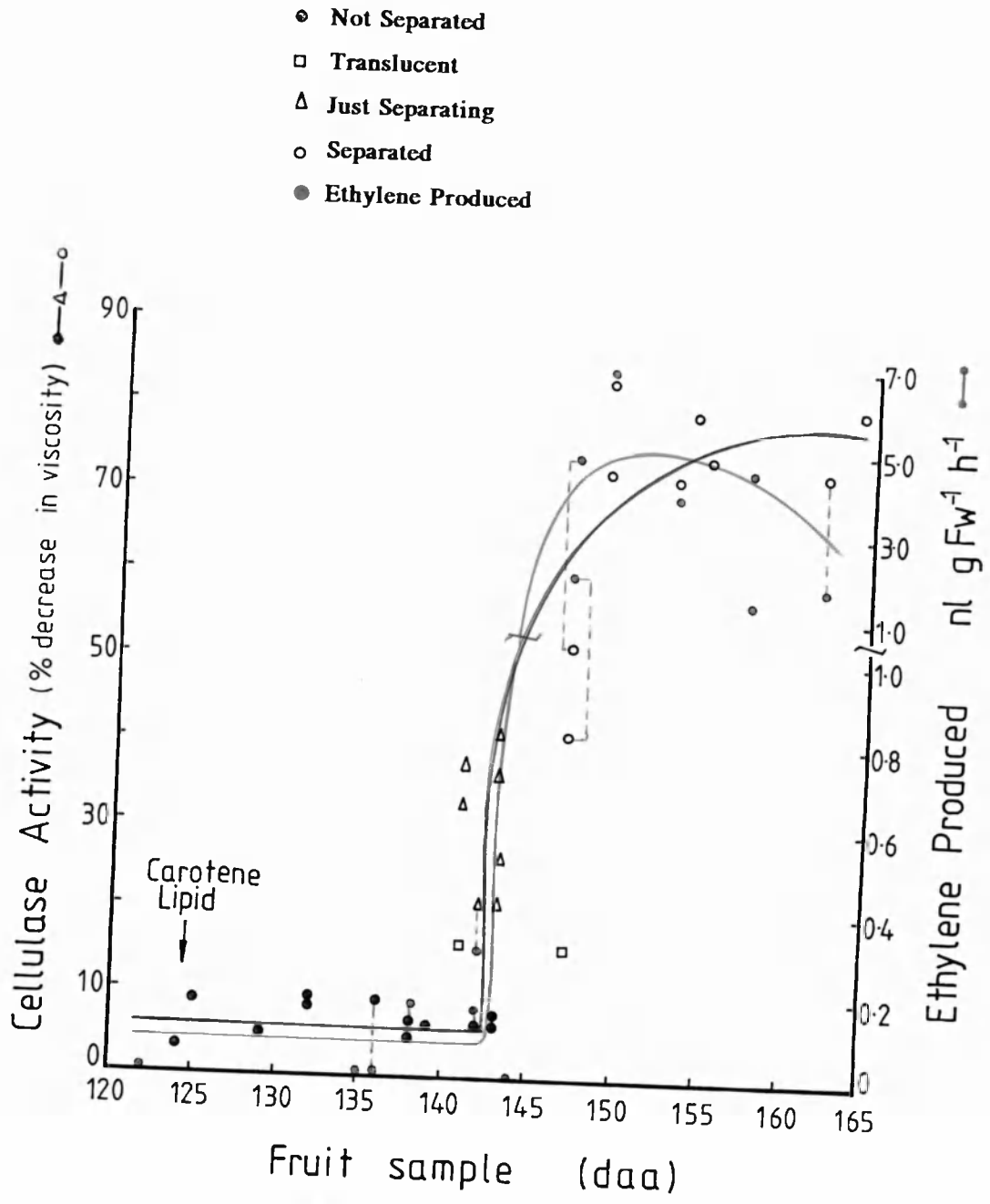


FIG. 5.2: CELLULASE ACTIVITY IN THE MESOCARP, ETHYLENE PRODUCTION OF THE FRUIT AND STAGE OF ABSCISSION

Mesocarp samples were taken from fruit on arrival in the laboratory (0h). Cellulase activity was measured (% decrease in viscosity in 2.5h for 100μl enzyme extract). The ethylene production in fruit was measured at 0h (30-60min closure periods). The broken line links the ethylene production and cellulase activity of the same fruit. A longitudinal section of the fruit after the ethylene measurement (Fig. 2.9) showed the stage of separation at Position 1.

was very low in phosphate buffer above pH 6.5. This was an effect of the higher pH, rather than of the phosphate ion. Phosphate buffer is inhibitory to a number of enzymes (Calbiochem, 1975) but mesocarp cellulase activity remained unchanged from that in acetate buffer when assayed in phosphate (pH 5.8) at concentrations of 100, 50, 10 and 1mM (not shown). At pH 6.8 mesocarp values were always lower than those of separated zone (Section 5.1.2).

The increase in mesocarp cellulase activity with fruit age was examined in ripening to fully ripe fruit between 124-164 daa (Fig. 5.2). The mesocarp samples for cellulase assay were collected and frozen (in liquid nitrogen) immediately on arrival in the laboratory (0h) and the stage of zone separation noted. In 9 different fruit samples, the ethylene production of each fruit was determined and then the mesocarp of this fruit collected and frozen for a cellulase assay. (These experiments were done in collaboration with Ms A. Norville).

Figure 5.2 shows that the rise in cellulase activity in the mesocarp closely parallels the timing of the increase in ethylene production and cell separation at Position 1. There was $<10\%$ decrease in viscosity assays (2.5h) by mesocarp extracts of ripening but unseparated fruit. When the zone became translucent or when separation at Position 1 had commenced, cellulase activity then increased from ~14% to over 40%. In all fully separated ripe fruit, viscosity loss of mesocarp extracts was always above 40% (40-83%).

Many attempts were made to visualise the cellulase protein of oil palm mesocarp after electrophoresis in activity gels. After incubation with CMC substrate, the gel was washed and stained with Congo Red. However, these activity gels were not successful. Since we were able to visualise the avocado cellulase by this method, the activity of the ripe mesocarp of oil palm was compared directly by a viscometric assay with that in avocado (Hass). Enzyme extracts of oil palm and avocado mesocarp were prepared and assayed in parallel (Section 2.6.3) and the activity of an abscission zone extract is included here for comparison (Table 5.1):

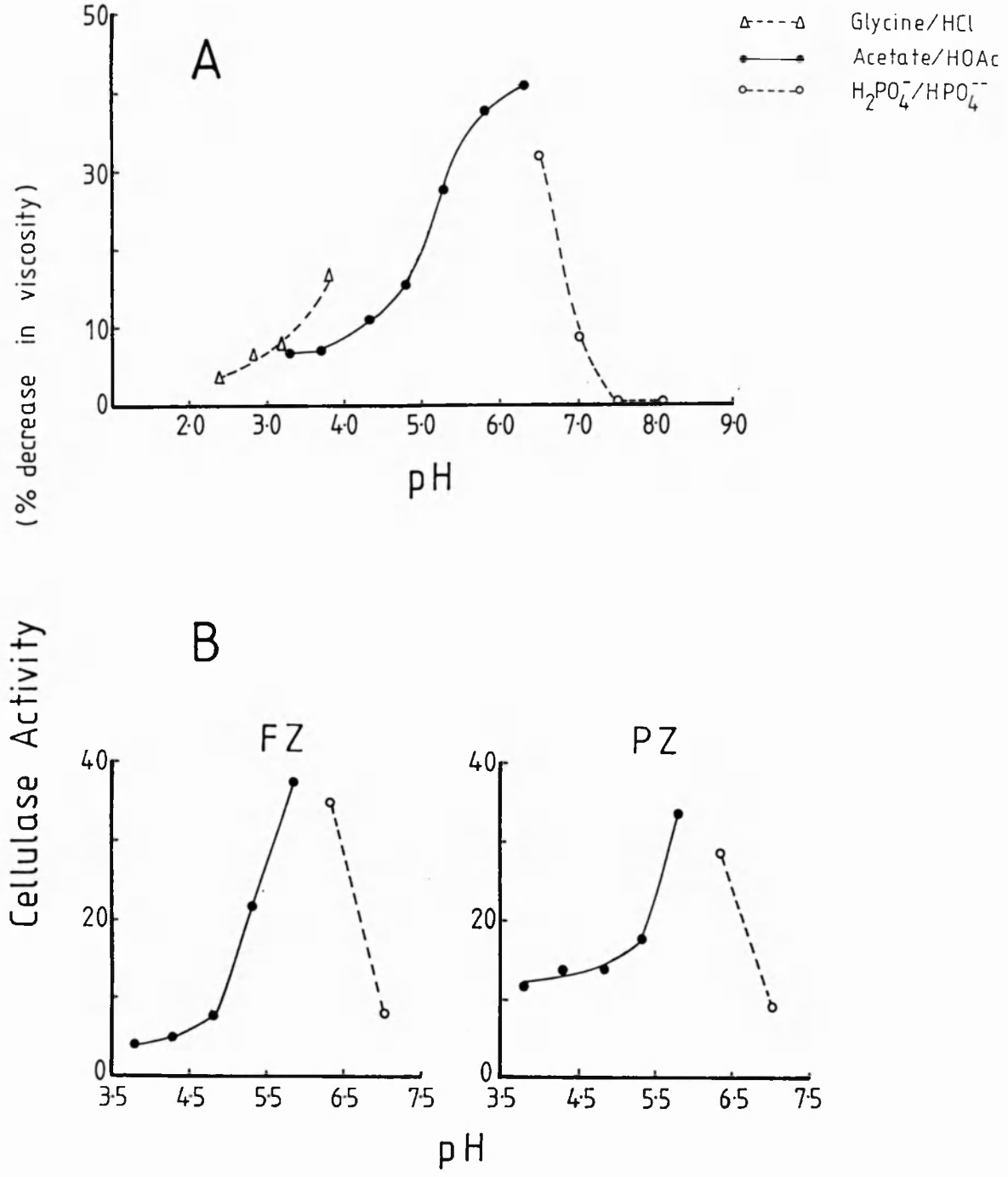


FIG. 5.3: THE PH-DEPENDENT ACTIVITY OF CELLULASE IN THE SEPARATED ABSCISSION ZONE

A. The cellulase activity of an enzyme extract of ripe normal separated zone (147 daa) (% decrease in viscosity in 3h for 50μl enzyme extract) was examined in three different buffers (100mM glycine, 100mM acetate and 100mM phosphate) between pH 2.4-8.1.

B. The cellulase activity of enzyme extracts of ripe normal separated FZ and PZ (152 daa) (% decrease in viscosity in 3h for 50μl enzyme extract) was examined in 100mM acetate and 100mM phosphate buffers between pH 3.7-6.8.

	% DECREASE VISCOSITY (30°C) 100mM Acetate pH 5.8				
	5min	15min	30min	1h	3h
AVOCADO MESOCARP					
50μl enzyme extract	77.4	89.4	95.2	-	-
10μl enzyme extract	17.4	66.1	80.4	-	-
1μl enzyme extract	-	-	26.4	46.3	69.3
0.5μl enzyme extract	-	-	10.9	25.3	46.8
0.1μl enzyme extract	-	-	8.5	15.0	21.9
OIL PALM MESOCARP					
50μl enzyme extract	-	-	17.3	49.3	77.9
OIL PALM SEPARATED ZONE FZ/PZ					
50μl enzyme extract	-	-	-	11.3	33.4

Table 5.1: Comparison of the Cellulase Activity in Ripe Avocado Mesocarp with that in Ripe Separated Oil Palm Mesocarp and Zone (FZ/PZ)

The activity of the cellulase(s) in oil palm mesocarp and zone is considerably lower (by 50-100 times) than in avocado mesocarp and this experiment indicates that in order to detect activity after electrophoresis, particularly with the zone extracts, oil palm cellulase needs to be purified and concentrated considerably (currently in progress).

5.1.2 Cellulase Activity in the Zone

Cellulase activity in ripening fruit was too low to detect in the unseparated zone, and in ripe fruit with unseparated but translucent zones the activity detected may have been due to contamination with mesocarp tissue when the zone slice is excised. However, separated zone (FZ/PZ) always contained cellulase activity. The pH-dependency of the zone cellulase was examined in the buffers: 100mM glycine/HCl (pH 2.4-3.6) 100mM acetate (pH 3.8-6.3) and 100mM phosphate (pH 6.5-8.1). Figure 5.3 shows that, in contrast to the mesocarp, activity in the zone was very low at more acidic pH. In phosphate buffer (pH 6.5-7.0) there appears to be slightly higher activity in the zone than in mesocarp, but most activity is in acetate buffer at pH 5.8. The FZ side of the zone was compared with the PZ side and there appears to be no difference in either activity or pH dependency.

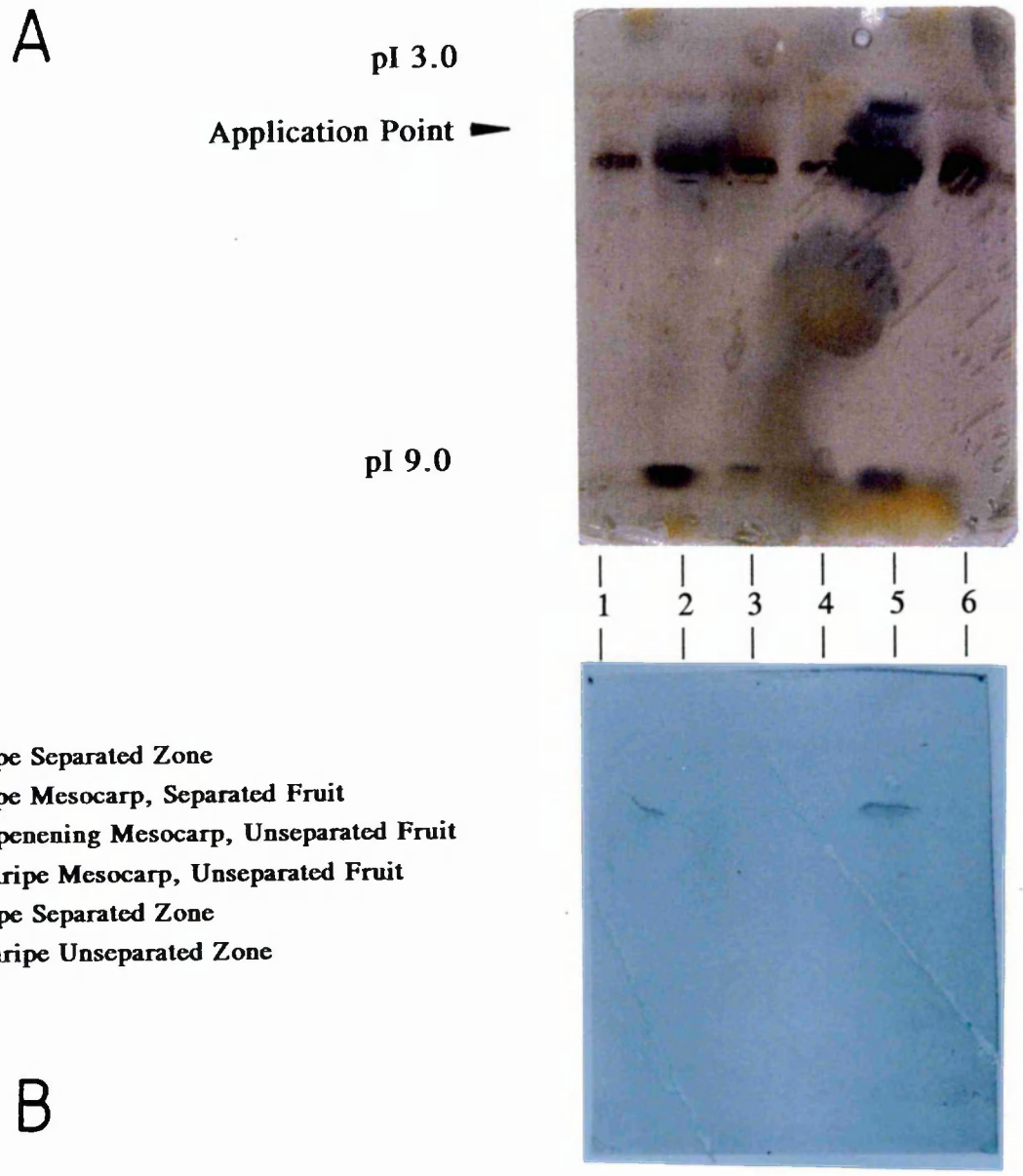


FIG. 5.4: IMMUNORECOGNITION OF OIL PALM CELLULASE WITH THE BEAN ABSCISSION CELLULASE PI 9.5 ANTISERUM

A. Enzyme extracts (4μl) of mesocarp (unripe; ripening; and ripe) and zone (unripe unseparated and ripe separated) were fractionated by IEF (Phastgel, pI 3.0-9.0) and after blotting (see B, below) was silver-stained for protein.

B. A Western blot of the above gel (A) probed with the bean abscission cellulase pI 9.5 antiserum. Recognition was obtained only with the separated zone extract at the point of sample application (~pI 4.5).

(Unripe = 106 daa; Ripening = 126 daa; Ripe = 158 daa.)

5.1.3 Immunorecognition of Mesocarp and Zone Cellulase

The different profile of pH dependency of mesocarp and zone cellulase suggests that they are not the same isoform. Neither a tomato nor an avocado cellulase antibody (both provided by Dr A. Bennett) recognised the oil palm mesocarp cellulase by ELISA (assayed by A. Norville) or on Western blots. This may have been due to the enzyme extracts being insufficiently concentrated.

The bean abscission cellulase pI 9.5 antiserum (a gift from Dr R. Sexton) did not recognise a mesocarp cellulase, and weak recognition was only obtained with separated zone extracts. Figure 5.4 shows that when extracts of ripe and unripe mesocarp, separated and unseparated zone were fractionated on an IEF gel (PhastGel) and probed with bean abscission cellulase pI 9.5 antiserum, recognition was obtained only in the extract from separated zone. This gives further support to the notion that the mesocarp and zone cellulases are different isoforms. (In this experiment, the zone abscission cellulase did not have a basic pI of 9.5 which may have been either because most of the cellulase enzyme was trapped in pectin and remained at the point of application, or because the oil palm abscission cellulase has a pI ~4.5.)

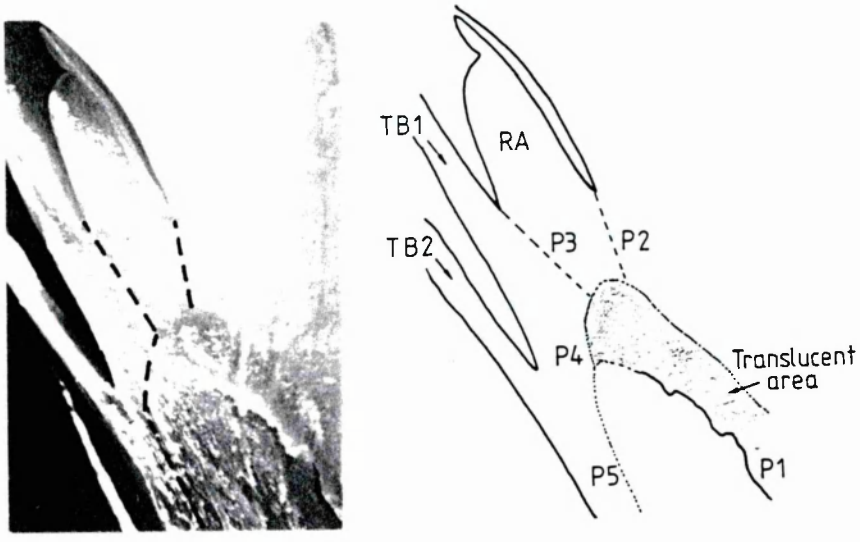
5.1.4 Cellulase Activity of the Fruit Periphery (Position 2/3)

Since the mesocarp and separated zone cellulases have a different pH dependency, it is possible to determine which cellulase type is present at Position 2/3 after fruit separation (1+2/3).

	% DECREASE IN VISCOSITY 2.5h	
Ripe, Separated (146 daa)	**pH 4.5**	**pH 6.3**
Zone FZ/PZ (Position 1)	9.0	52.9
Periphery (Position 2/3)	6.6	53.0
Mesocarp	42.3	63.3

Table 5.2: Cellulase Activity at pH 4.5 (50mM Acetate) and pH 6.3 (50mM Phosphate) in Separated Zone, Fruit Periphery and Mesocarp Extracts.

There is low activity at pH 4.5 as there is in the zone, whereas the mesocarp has high activity at this pH (Table 5.2). Thus, the cellulase activity in extracts of the fruit periphery (Position 2/3) is "zone" type.



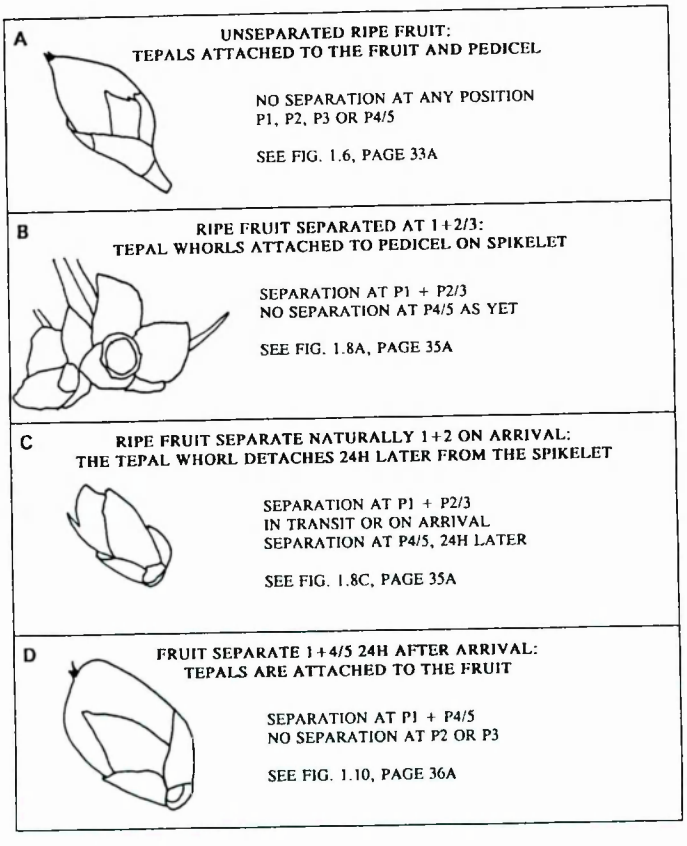


FIG. 5.5: THE POSITIONAL SITES INVOLVED WITH TEPAL SEPARATION AND DIAGRAMS SHOWING EACH TYPE OF TEPAL COLLECTED FOR TISSUE SAMPLES:

A & B TEPALS UNSEPARATED FROM PEDICEL

C & D TEPALS SEPARATED FROM PEDICEL

5.1.5 Cellulase Activity of the Tepal Base (Positions 2/3 and 4/5)

When fruit separate naturally 1+2/3 it is between the fruit base and the RA/tepal base 1 (see Fig. 5.5). In Fig. 5.5B, tepal base 1 has not separated from the pedicel as yet and so the tissue near the base of the tepal represents Position 2/3 - the corresponding side of the fruit periphery. This tissue can be collected by removing tepal base 1 with force (that is, pulling it out of the empty fruit socket). Cellulase activity was generally difficult to detect in enzyme extracts of tepal base 1 in contrast with its corresponding face (the fruit periphery) (Table 5.2). In 9 different samples of ripe fruit which had separated 1+2 (Fig. 5.5B), the cellulase activity was 7.2 $\pm$ 1.8% (decrease in viscosity in 2.5h, 100μl of extract). Occasionally, up to a 20% decrease in viscosity (2.5h) was found which may have been due to pieces of zone tissue adhering to the base of the tepal. In order to determine if cellulase was induced in the tepal base after 1+2 fruit separation (Fig. 5.5B), an increased amount of enzyme extract was used (200μl in 2.5% CMC solution) and incubated for a longer period (20h). However, gelling reactions of the pectin in the extract increased the viscosity of the CMC solution and masked the actual cellulase activity which may have been present.

Of the positions of separation shown in Fig. 5.5, cellulase activity was detected in relatively high levels only in Position 1; possibly similar levels at Position 2/3 after natural fruit abscission (1+2/3). The question arises, is cellulase activity involved at Position 4/5 during separation of the tepal bases?

The two types of tepal separation from the pedicel were compared (Fig. 5.5C & D). When a fruit has separated 1+2 the cup of tepals remains on the spikelet and the tepal bases are attached to the pedicel (Fig 5.4B). In the field, this cup of tepals separates from the sides of the pedicel (Position 4/5) about 24h after fruit abscission, and can be shed as a complete unit (Fig. 5.5C). In contrast, in the laboratory, separation at Position 4/5 more usually occurs simultaneously with Position 1 separation, so that the fruit is then shed with tepals attached (Fig. 5.5D). There was no difference in the cellulase activity of tepal base 1 in these two types of separation (Fig. 5.5C & D). These results are shown in Table 5.3.

- **Tepal Base 1**

The level of activity in tepal base 1 is very much lower than at Position 1 (separated zone). Cellulase activity was at least 8 times greater in the zone than in tepal base 1 when comparable amounts of enzyme extract were assayed for 2.5h. In addition, the cellulase activity of pedicel fibres was found to be similar to that in tepal bases. Table 5.3 includes for comparison the activity in separated zone (Position 1) and pedicel tissue with that found in tepal base 1.

	% DECREASE IN VISCOSITY pH 5.8	
	200µl of Enzyme Extract	
TEPAL BASE 1 OF RIPE FRUIT	**2.5h**	**20h**
No separation at zone, tepals attached to fruit and pedicel, Fig. 5.5A (n = 3)	5.1 ± 0.8	21.3 ± 8.4
Tepal whorl separates from pedicel (4/5) 24h after fruit abscission, Fig. 5.5C (n = 2)	7.7 ± 0.1	27.4 ± 1.0
Fruit separates from spikelet 1+4/5, tepals attached to fruit, Fig. 5.5D (n = 2)	7.9 ± 0.5	27.7 ± 0.7
SEPARATED ZONE FZ Position 1 (n = 1)	68.0	-
PEDICEL FIBRE TISSUE (n = 2)	nd*	15.5 ± 6.2

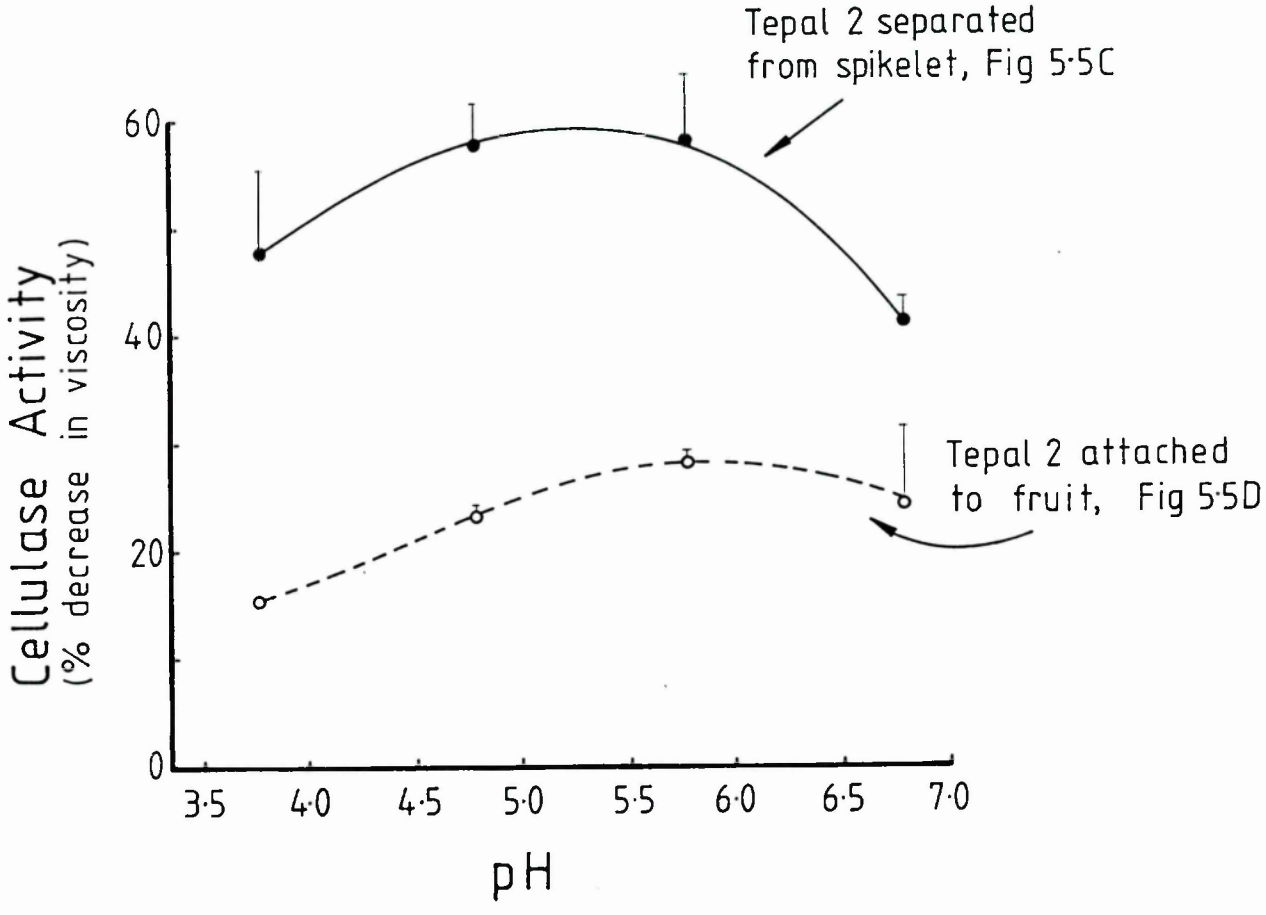
nd* = not detected

Table 5.3: Cellulase Activity in Tepal Base 1 of Ripe Fruit as shown in Figure 5.5A, C & D: Tepals Attached to Unseparated Fruit (A); Tepals Separated from Pedicel 4/5 (C); and Tepals Attached to Fruit after Separation from Pedicel 1+4/5 (D). Activity in Extracts of Separated Zone and Pedicel Tissue are Included for Comparison.

These results indicate that the cellulase activity in tepal base 1 is not induced to high levels when fruit separate or when the tepal itself separates from the pedicel.

- **Tepal Base 2**

Tepal 2 has no attachment to the fruit at all and after separation the base of tepal 2 represents Position 5. As described for tepal base 1 above, the two types of separation from the pedicel were compared. Cellulase activity was examined in tepal base 2:



5.6: THE pH-DEPENDENT ACTIVITY OF CELLULASE IN TEPAL BASE 2 (POSITION 5)

Tepal base 2 from ripe normal fruit (147 daa) which separated 1+4/5 (48h after arrival in the laboratory, Fig. 5.5D) and tepal base 2 from the whorl of tepals which separated from the pedicel (Fig. 5.5C) were extracted as described in Section 2.6.1. Cellulase activity (% decrease in viscosity in 20h for 200μl of enzyme extract) was determined in 100mM acetate buffer pH 3.8-5.8 and 100mM phosphate buffer at pH 6.8.

(i) after the fruit has shed, and the cup of tepals remaining on the spikelet separates from the pedicel and is shed as a complete whorl (Fig. 5.5C).

(ii) from fruit which separate 1+4/5 with the tepals attached to the fruit (Fig. 5.5D).

The cellulase activity in tepal base 2 of fruit which separate 1+4/5 (with tepals attached, Fig. 5.5D) was similar to that found in tepal base 1 (Table 5.3). However, the activity in tepal base 2 from the whorl of tepals which separate from the pedicel after fruit abscission (Fig. 5.5C) was at least twice that found in tepal base 1 and tepal base 2 from 1+4/5 separated fruit (Table 5.4).

	% DECREASE IN VISCOSITY pH 5.8	
	200µl of Enzyme Extract	
TEPAL BASE 2 OF RIPE FRUIT	**3h**	**24h**
Tepal whorl separates from pedicel (4/5) 24h after fruit abscission, Fig. 5.5C (n = 2)	16.4 ± 4.2	56.7 ± 8.0
Fruit separates from spikelet 1+4/5, tepals attached to fruit, Fig. 5.5D (n = 2)	7.7 ± 2.8	28.5 ± 3.0

Table 5.4: Cellulase Activity in Tepal Base 2 of Ripe Fruit as shown in Figure 5.5C & D: Tepals Separated from Pedicel 4/5 (C); and Tepals Attached to Fruit after Separation from Pedicel 1+4/5 (D).

The pH dependent activity was compared between pH 3.8 and 6.8 (Fig 5.6) and was similar to the pH profile of the mesocarp-type cellulase (Fig. 5.1), with relatively high activities at lower pH values but unlike the mesocarp-type, activity was obtained at pH 6.8. The zone-type had some activity at pH 6.8. Therefore, the activity in tepal bases may represent the combined activities of both mesocarp-type and zone-type cellulases or it may be another cellulase isoform specifically expressed in tepal base separation.

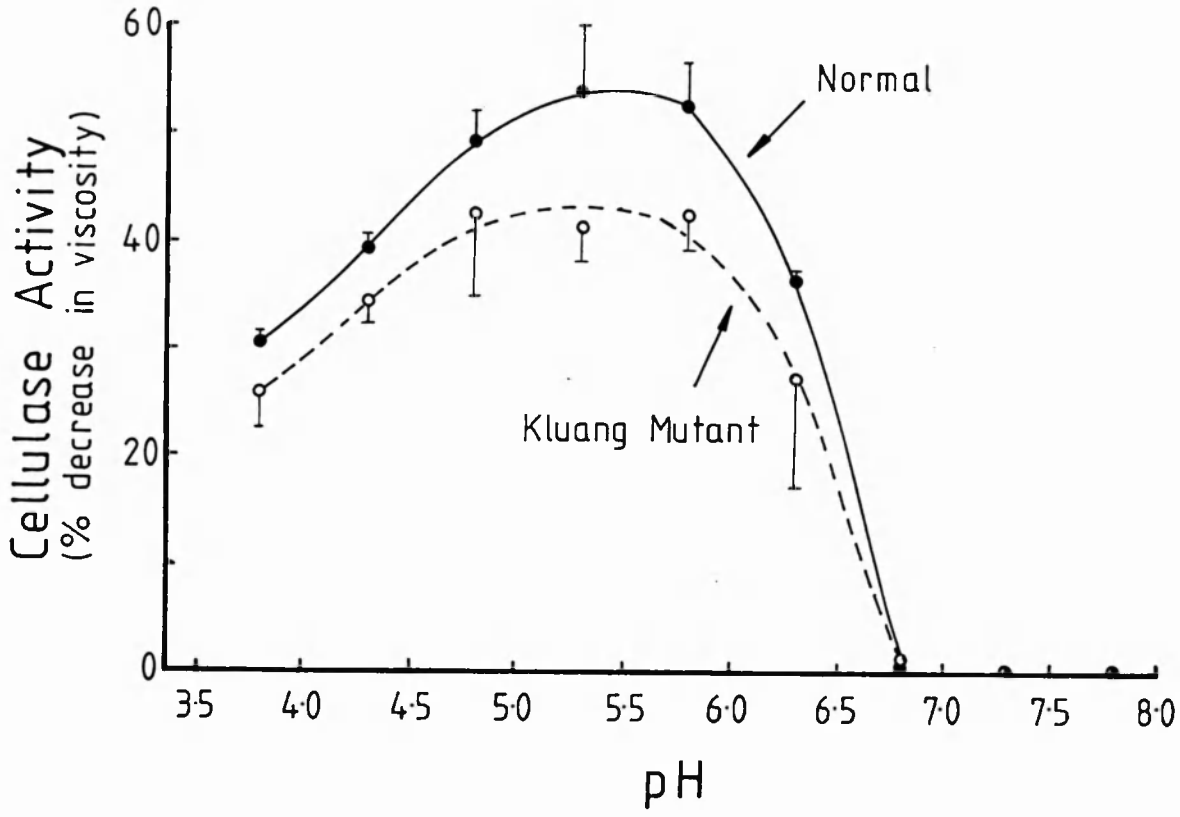


FIG. 5.7: THE PH-DEPENDENT CELLULASE ACTIVITY IN THE MESOCARP OF NORMAL AND KLUANG MUTANT FRUIT

The cellulase activity (% decrease in viscosity in 1h for 50μl enzyme extract) was examined between pH 3.8-7.8 (100mM acetate and 100mM phosphate buffer) in ripe normal mesocarp (n = 2) and ripe Kluang mutant mesocarp (n = 2).

5.2 A COMPARISON OF CELLULASE ACTIVITY IN NORMAL AND KLUANG MUTANT FRUIT

5.2.1 Comparison of Cellulase Activity in the Mesocarp

The pH-dependent assays of enzyme extracts of mesocarp in the normal and Kluang mutant fruit confirmed that most activity is obtained at pH 5.3 to 5.8 in acetate buffer but activity even at pH 3.7 is high. Figure 5.7 shows that the pH profile of both the normal and Kluang mutant is essentially the same for the mesocarp tissue.

The cellulase activity of the "gelatinous" mesocarp of damaged Kluang mutant fruit was examined and found to have very high cellulase activity. Fruit which had both gelatinous and non-gelatinous tissue within the same fruit were compared (Table 5.5).

Kluang Mutant (218 daa)	% Decrease in Viscosity 2.5h pH 4.5
Gelatinous Mesocarp (n = 4)	89.4 ± 1.3
Non-Gelatinous Mesocarp (n = 4)	58.6 ± 10.2

Table 5.5: Cellulase Activity in the Mesocarp of Damaged Kluang Mutant Fruit

The greater activity in the gelatinous mesocarp region may result from changes in compartmentalisation within the cells which leads to cellulase being more easily extracted or, possibly, more enzyme may be synthesized by the damaged tissue.

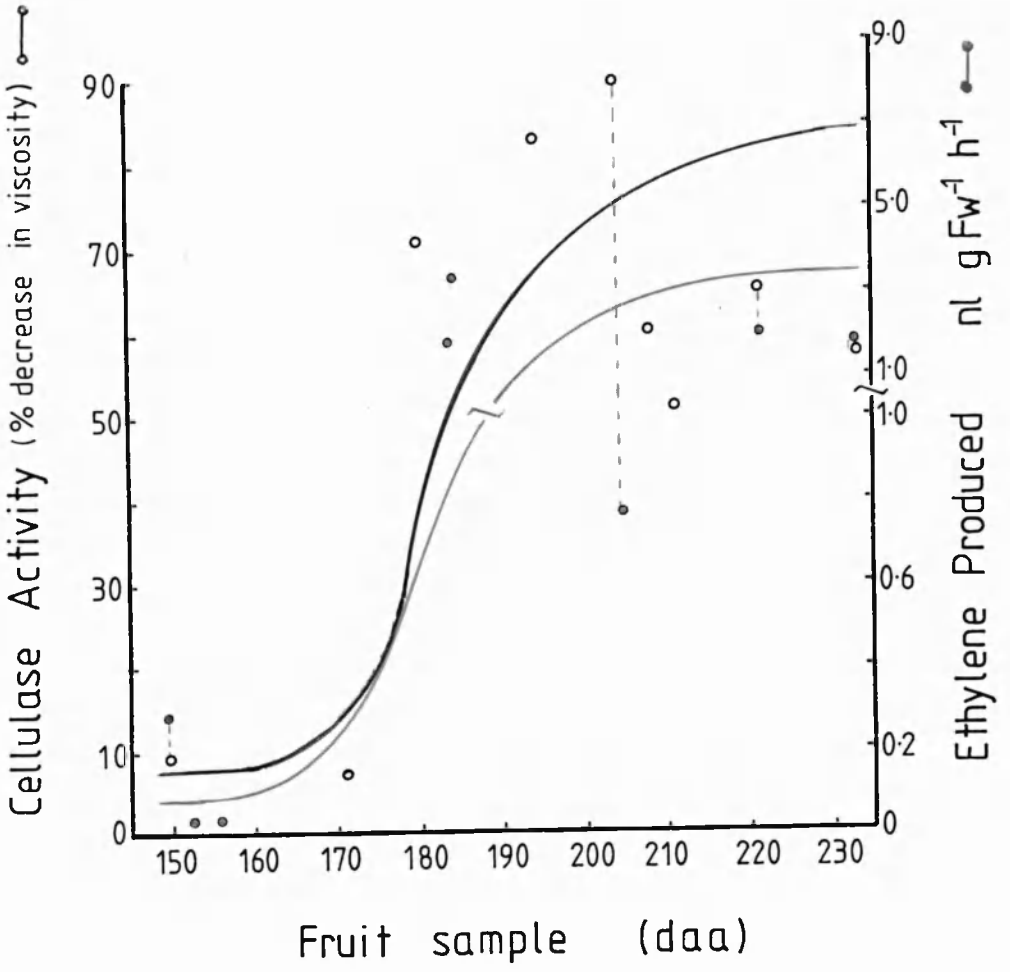


FIG. 5.8: CELLULASE ACTIVITY IN KLUANG MUTANT MESOCARP AND ETHYLENE PRODUCTION OF FRUIT BUT NO SEPARATION HAS OCCURRED AT THE ABSCISSION ZONE

Mesocarp samples were taken from fruit on arrival in the laboratory (0h). Cellulase activity was measured (% decrease in viscosity in 2.5h for 100μl enzyme extracts). Ethylene production in fruit was measured at 0h (30-60min closure periods). The broken line links the ethylene production and cellulase activity of the same fruit. A longitudinal section of the fruit (Fig. 2.9) after the ethylene measurement showed no separation had occurred at Position 1.

In normal fruit mesocarp, cellulase starts to increase in activity only when ripe fruit start to produce ethylene, just before abscission (see Fig. 5.2). That is, ethylene production, mesocarp cellulase activity and fruit separation appear to be linked.

A different situation exists with the non-abscinding Kluang mutant. Whereas ethylene production and cellulase activity are still closely correlated, and rise at the same time, these events are not the immediate prelude to abscission.

On arrival in the laboratory, cellulase activity and ethylene production were examined in the unseparated ripe fruit of the Kluang mutant (Fig. 5.8). In mesocarp of fruit 150-156 daa, only very low cellulase activity and ethylene production could be detected; as the fruit continued to the over-ripe stage, cellulase activity increased to high levels coincident with the rise in ethylene production.

Table 5.6 shows that even though there were high levels of cellulase activity in the mesocarp and ethylene production too is high, separation at the abscission zone did not occur in the field or until at least 48h after arrival in the laboratory.

Kluang Mutant Time of Arrival	**nl C_2H_4 Produced g Fw^{-1} h^{-1}**	**Mesocarp Cellulase Decrease in Viscosity 2.5h**	**Separation at Abscission Zone**
0h (n = 6)	-	83.9 ± 7.0	NO separation
24h (n = 6)	0.8 ± 0.3	88.9 ± 3.4	NO separation
72h (n = 3)	3.6 ± 1.1	85.1 ± 1.6	Separated
120h (n = 1)	-	86.5	Separated

Table 5.6: Mesocarp Cellulase Activity, Ethylene Production and Separation Stage of the Zone in Kluang Mutant Fruit (194 daa)

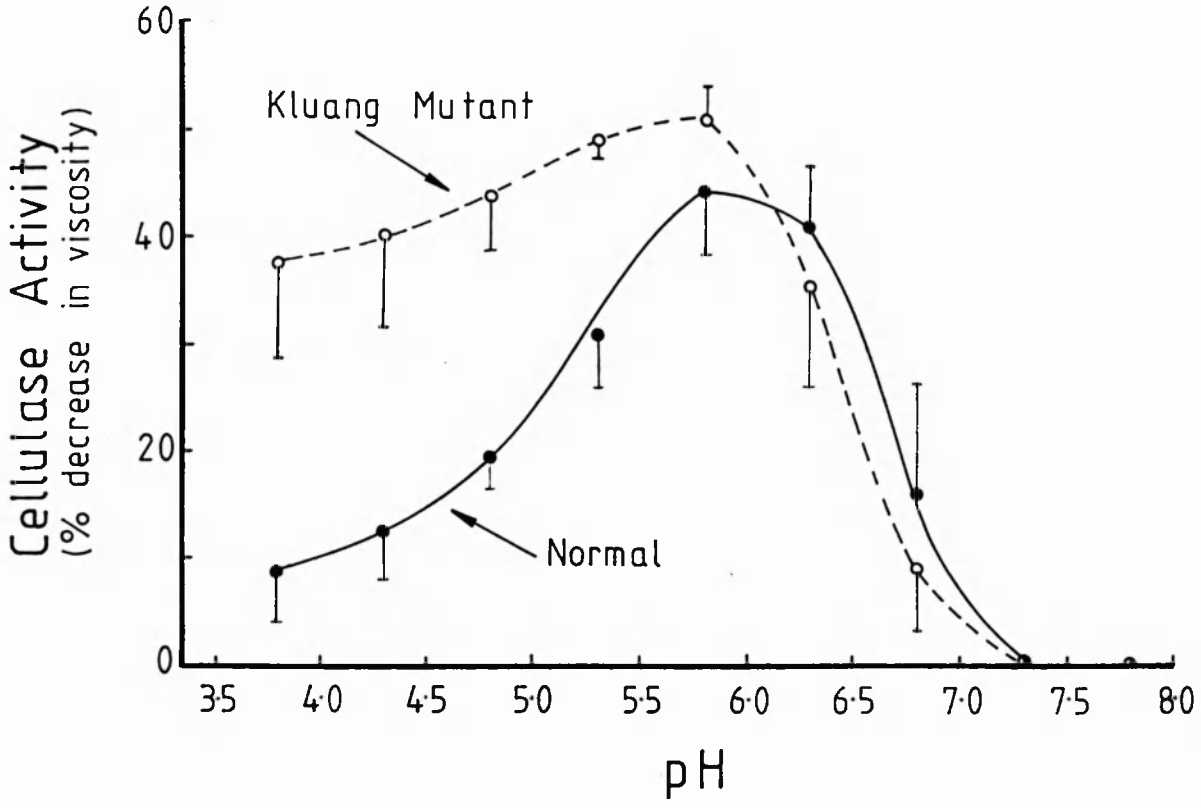


FIG. 5.9: THE PH-DEPENDENT CELLULASE ACTIVITY IN THE SEPARATED ZONE OF NORMAL AND KLUANG MUTANT FRUIT

Cellulase activity (% decrease in viscosity in 3h for 50μl enzyme extract) was examined between pH 3.8-7.8 (100mM acetate and 100mM phosphate buffer) in ripe normal separated zone (n = 3) and the ripe Kluang mutant separated zone (n = 5).

5.2.2 Comparison of Cellulase Activity in the Separated Zone

Whereas cellulase activity of the normal separated zone was low when assayed between pH 3.7 and 4.8, with maximum activity at pH 5.5-6.0 (see also Fig. 5.3), that of the Kluang mutant zone, which separated in the laboratory, showed high activity over the whole range from pH 3.7-6.3 (Fig. 5.9).

In comparative assays at pH 4.3-4.5 of enzyme extracts from normal separated zone and those of laboratory-separated Kluang fruit, cellulase activity was never greater than 17.7% (decrease in viscosity) in normal zones or, less than 30.1% (decrease in viscosity) in the Kluang mutant (Table 5.7).

	% DECREASE VISCOSITY 2.5h at pH 4.3-4.5	
Time after Arrival	**Normal**	**Kluang Mutant**
0h	**13.0** (151 daa)	**33.0, 30.1** (212 daa); **58.8** (233 daa)
24h	**9.0** (146 daa); **12.1, 17.7, 8.4** (147 daa)	**43.1** (212 daa)
72h	**11.0** (148 daa)	**37.3** (194 daa); **43.1, 51.4** (201 daa)
120h	-	**48.1** (194 daa)
Mean ± Std Dev	**11.9 ± 3.4** (n = 6)	**43.1 ± 9.6** (n = 8)

Table 5.7: **Cellulase Activity in Enzyme Extracts of Normal and Kluang Mutant Separated Zone. Tissue Samples were Collected when Fruit Separated After Arrival in the Laboratory at Various Times.**

The statistic for the difference between the two means in a small sample (t-distribution, $\alpha = 0.01$) showed clearly that the cellulase of the Kluang mutant separated zone is significantly different from that in the normal ($142.6 > 2.7$ with 12 df). In fact, with respect to its pH range of activity, it resembles the mesocarp cellulase (Fig. 5.7).

5.3 SUMMARY AND DISCUSSION

5.3.1 Cellulase Activity in the Abscission Zone

- **Position 1**

The zone-type cellulase from Position 1 was detected in enzyme extracts of separated ripe zone tissue and its activity in viscosity assays was always lower than the mesocarp type (Table 5.1). The zone-type cellulase, but not the mesocarp-type enzyme, also occurs in separation at Position 2/3 (Table 5.2). Thus, natural abscission (1+2/3) occurs after the induction of a specific zone cellulase. There was some recognition of the zone-type cellulase (but not the mesocarp-type) with the bean abscission pI 9.5 cellulase antiserum (Fig. 5.4).

Cellulase activity in separated zone extracts of the normal and Kluang mutant was similar at the optimum pH of 5.8. However, when assayed at different pHs (3.8-6.8) a very interesting difference was revealed. At pH 4.3-4.5 activity was significantly higher in the Kluang mutant (Table 5.7). The pH profile of the Kluang mutant (between pH 3.8-5.8) resembled that obtained for the extracts of mesocarp but this does not necessarily mean that this mesocarp enzyme is that induced in the Kluang mutant zone. The mutant enzyme may be "disabled" in some way resulting in the similar pH profile. It is not known if, at full ripeness whilst the fruit is still on the mutant palm, the zone cellulase is ever synthesized but if it is, it must be either unstable or unable to effect the subsequent changes which result in separation. Certainly, a zone cellulase was never detected on arrival in the laboratory (24h-30h post-harvest). After harvesting, conditions change. Either some "disabled" cellulase is then synthesized or a different post-harvest isoform ("late" cellulase) becomes operational. The environment of the zone cell walls may also change. A combination of these events then leads to a slow fruit separation.

Plant cellulases or endo-glucanases (EGases) are encoded by multigene families. In tomato they consist of at least 6 members each possessing a distinctive pattern of temporal and tissue specific expression (del Campillo & Bennett, 1996; Brummell *et al*, 1997). This is demonstrated in tomato flower abscission where the expression of specific cellulase mRNAs has been shown to be different in natural abscission (*in planta*) compared with that in explants (del Campillo & Bennett, 1996). These authors suggest that if multiple cellulases are required to cause abscission in tomato flowers the relative importance of each cellulase in the process may depend on the physiological conditions

under which abscission takes place. It is, therefore, possible that the changed physiological conditions after harvesting in the Kluang mutant fruit causes the induction of a different cellulase gene which results in separation ("late" cellulase).

Other research with the non-softening *rin* tomato is also relevant. Although the *rin* mutant has near wild-type levels of extractable CMCase activity (Brummell *et al*, 1994), the mRNA levels of one of the cellulase genes is almost absent, even with ethylene treatment. Gonzalez-Bosch *et al* (1996) suggest that the product of the *Cel*2 gene contributes to ripening-associated cell wall changes. Harvested *rin* fruit do accumulate a low level of PG mRNA (2.6% of wild-type levels) identical in size to wild-type (DellaPenna *et al*, 1987) but PG activity is low. They do not soften even when they have a PG transgene and are treated with ethylene or propylene. Perhaps the activity of a specific cellulase isoenzyme, for example the product of the *Cel*2 gene in tomato, precedes PG activity and subsequent PG induction is dependent upon (in addition to ethylene) the prior action of the specific cellulase isoenzyme. In fact, DellaPenna *et al* (1987) suggested that PG gene expression may be regulated, at least in part, by some developmental cue other than increased levels of ethylene. The separation of the harvested Kluang mutant fruit also suggests such a sequence. In addition, the "Sabah Palm 2", one of the 3 mutant palms discovered in the Sabah plantation, appears to have the same lesion in the abscission zone cellulase isoenzyme as the Kluang mutant. The pH-dependent profile was similar to that of the Kluang mutant, with high activity at low pH (data not shown).

- **Position 2/3**

Figure 5.5 shows the translucent area of the abscission zone at Position 1, but this translucency does not spread into Positions 2/3 when fruit separate 1+4/5 (Fig. 5.5D) and no cell separation occurs at Position 2/3 in the Kluang mutant. A possible explanation for this could be that, in normal fruit on the palm which separate naturally (1+2/3), the cellulase produced in Position 1 diffuses into Position 2/3, assisted by the transpirational flow. We recorded a considerable loss of water (5% decrease in weight per day) in each ripe fruit spikelet (Table 4.10, page 108). The rate of transpiration in oil palm has been measured by Jeje *et al* (1978). These authors report that fruit have 5-18 stomata per mm^2 (basal to apical region) and that in the last 3 weeks of ripening (at the same time as TAG biosynthesis) the transpiration rate increases by 2.5 times. They also showed that increased transpiration attended increased total oil formed. The inner fruits embedded

deep in the fruit bunch transpire less than outer fruit and as a consequence have less oil. This also relates to fruit abscission. As discussed in Section 1.3.2, on the palm, the outer fruit of the bunch abscind first and the central and inner fruit separate many days later.

The Kluang mutant appears to express either a "disabled" zone-type or a "late" cellulase which becomes active at Position 1 only after the fruit are harvested. Since separation does not usually occur at Position 2/3 in the mutant, fruit must be pressed off to obtain Position 1 zone samples. Harvesting the spikelets no doubt affects the transpirational flow, possibly reducing it, and as a consequence the diffusion of zone cellulase from Position 1 to Position 2/3 could be restricted. Even post-harvest normal fruit which are not close to natural abscission do not separate at Position 2/3. Position 2/3 is, therefore, a critical tissue involved in the natural separation of oil palm fruit. It is the final point of detachment of the fruit from the spikelet. The enzymes involved in cell separation here may not necessarily be synthesized in this tissue but could diffuse into Position 2/3 from their sites of synthesis in Position 1. This would then allow for the coordinated release of the fruit. Alternatively, a different composition and structure of the cell walls/middle lamella at Position 2/3 may require only low levels of hydrolytic enzymes. The abscission of the tepals remaining on the spikelet then follows which involves separation at Position 4/5 (Fig. 5.5).

- **Position 4/5**

Cellulase activity at Position 4/5 examined in tepal base 1 and 2 was very low; up to 10 times lower than activity measured in Position 1 (Tables 5.3 and 5.4). Polygalacturonase and glycosidase activities were also very low (Sections 6.2.1 and 8.1). What causes the separation of the tepals? Possibly, the same signal for fruit separation which is perceived by Position 1 abscission zone cells also initiates cell separation at Position 4/5. Possibly also, the low amount of cellulase and PG enzyme and/or the lower activities of other enzymes means a longer time period is required (than for Position 1) to achieve full separation of the tepal whorl unit. Another explanation may be that the cell walls at 4/5 are different from Position 1 walls and cell separation at Position 4/5 requires the involvement of other enzyme activities than cellulase and PG.

When fruit are harvested which are not close to abscission, separation at Position 1 and Position 4/5 is simultaneous; the tepals are attached to the shed fruit (Fig. 5.5D). This does not occur on the palm and the tepals cannot be removed from the spikelet until

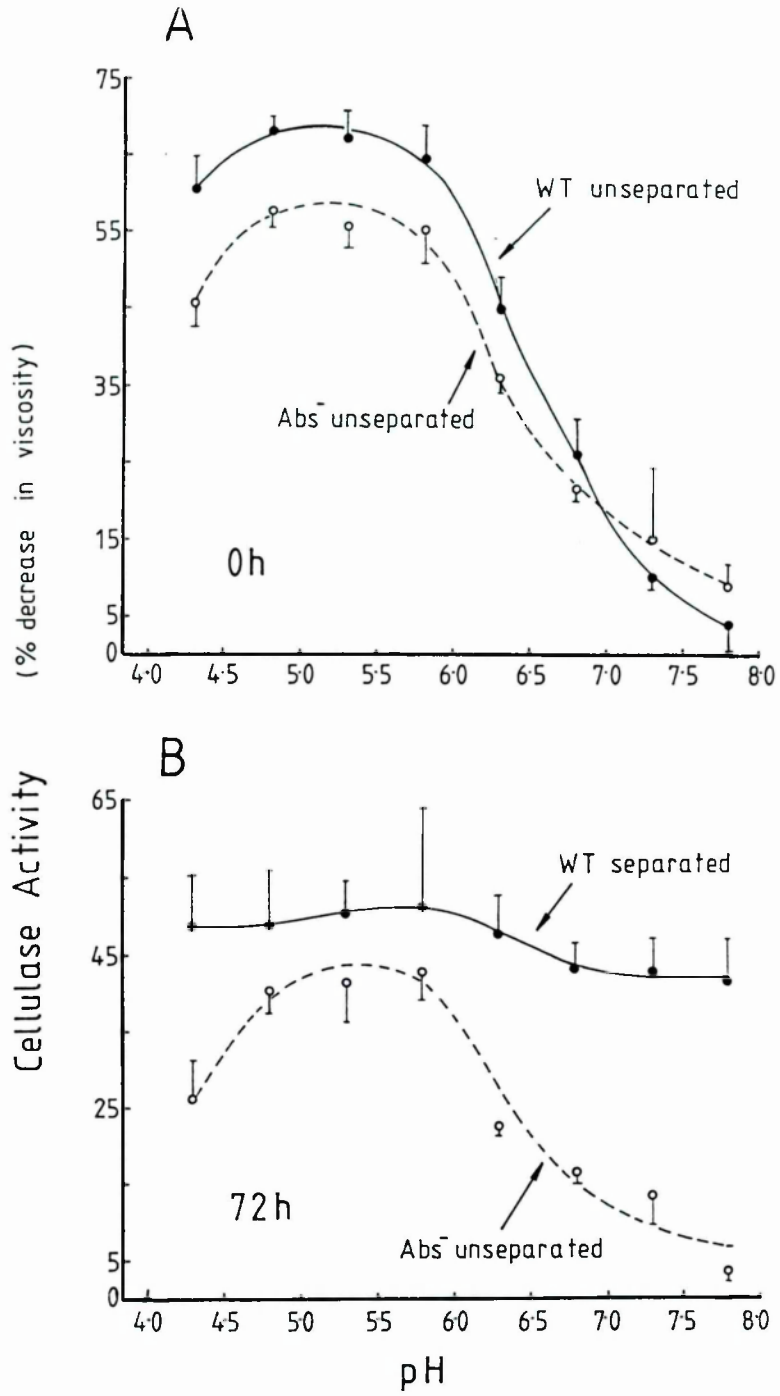


FIG. 5.10: THE pH-DEPENDENT CELLULASE ACTIVITY OF THE LUPIN LEAF-PETIOLE ABSCISSION ZONE.

A. The abscission region from the unseparated wild-type and *Abs*⁻ leaf-petiole was excised from the plant at 0h. The cellulase activity (% decrease in viscosity in 3h for 40μl enzyme extract) was examined between pH 4.3-7.8 (100mM acetate and 100mM phosphate buffer).

B. The separated abscission zone of the wild-type and the unseparated abscission region of *Abs*⁻ from leaf-petiole explants which were treated with 5μl l^{-1} ethylene for 72h (with aeration every 24h). Cellulase activity (% decrease in viscosity in 3h for 40μl enzyme extract) was examined between pH 4.3-7.8 (100mM acetate and 100mM phosphate buffer).

some hours after fruit separation (Section 2.2.1, page 44). For tepal base 1, whether tepals separate attached to the fruit (1+4/5) or remain on the spikelet and so separate naturally from the pedicel, the same low level of cellulase activity is obtained at Position 4/5 (Table 5.3). However, surprisingly, cellulase activity in the base of tepal 2 (Position 5) from the tepal whorl which separates naturally from the pedicel 24h after fruit abscission (although still low in comparison with activity in the mesocarp and zone) was over twice that found in fruit which separate 1+4/5 (with the tepals attached to the shed fruit) (Table 5.4). The pH profile and levels of activity in the two types of tepal 2 separation may suggest the action of different cellulase enzyme(s) (compare Figs. 5.1, 5.3 and 5.6).

5.3.2 Cellulase Activity in the Zone of a Non-Abscinding Dicotyledon

For comparison with the oil palm, the leaf-petiole zone of a non-abscinding mutant of *Lupinus angustifolius* (the fodder lupin) has been investigated and here too a "disabled" or inactive cellulase appears to be involved. After incubation of leaf explants in an ethylene atmosphere (72h), Osborne & Thompson (1991) found cellulase activity (pH 4.5) in separated wild-type zones but in the non-abscinding mutant (*Abs*$^-$) there was only 19% of wild-type activity. Subsequent experiments using a different extraction method (based on that developed for the extraction of oil palm tissue) examined the pH dependent cellulase activity at 0h and after 72h in ethylene (5μl l^{-1}) in both wild-type and *Abs*$^-$ zone tissue. The tissue was ground with acid washed sand and extracted (2x) with 1M NaCl. With these extraction conditions, a cellulase activity was now shown to be present in unseparated 0h zones of both wild-type and *Abs*$^-$, with most activity between pH 4.3-5.8 (Fig. 5.10A). In the 72h ethylene-treated tissue, wild-type leaf-petiole zones separate (100%) but those of the *Abs*$^-$ do not (0% abscission). The pH-dependent cellulase activity of 72h ethylene-treated *Abs*$^-$ was unchanged but in wild-type, activity was now obtained at the higher pHs of 6.3-7.8 as shown in Fig 5.10B. This suggests that, in addition to a constitutive cellulase, another isoenzyme had been induced at abscission with similar levels of activity over a higher pH range. In the bean petiole-leaf abscission zone, del Campillo *et al* (1988) showed the pH profiles for both the membrane-bound and soluble cellulases (including the abscission pI 9.5 cellulase) and these are very similar to the pH profile for the separated zone of the wild-type lupin, as shown in Fig. 5.10. This is not unexpected since the lupin and bean are in the same family (Leguminosae). Recognition was obtained in an ELISA of both wild-type separated and *Abs*$^-$ unseparated zone extracts with the bean

pI 9.5 cellulase antiserum after ethylene treatment (Thompson, 1991, and assays by A. Norville, 1994), indicating that the abscission cellulase protein is synthesized in both but this protein does not have abscission-related cellulase activity in the *Abs*⁻.

5.3.3 Cellulase Activity in the Oil Palm Mesocarp

Cellulase activity in the ripe separated oil palm fruit is only 1-2% of that in the ripe and soft avocado, even though both are oil-bearing fruit. The oil palm fruit does not soften like the avocado, possibly due in part to the greater amount of fibrous tissue present (Table 3.1, page 80). However, both fruit are similar with respect to the link between mesocarp cellulase activity and ethylene production.

In the avocado an increase in the cellulase activity starts soon after harvest and reaches high levels 8-10 days after harvesting; the ethylene production follows the same pattern (Awad & Young, 1979). In the oil palm mesocarp, the synthesis of cellulase and ethylene in parallel is also observed but this starts only at fruit separation (Fig. 5.2). Cellulase induction in the oil palm mesocarp is **not** linked to ripening, as it is in the avocado. Cellulase activity is barely detected as fruit start to ripen at ~125 daa, and for at least another 30 days, whilst carotene, lipase, TAG biosynthesis all increase dramatically (Section 1.3.1 and Fig. 1.5, pages 30-33).

The non-abscinding Kluang mutant fruit produces ethylene with a similar level of mesocarp cellulase activity. However, unlike normal fruit, ethylene production and the mesocarp-type cellulase activity are uncoupled from abscission.

* * * * * * *

In conclusion, two types of cellulase activity were detected in the oil palm fruit; an abscission-type in the zone (Positions 1 and 2/3) and a mesocarp-type. Cellulase was detected in the mesocarp, zone and tepal bases of separated fruit only. Most activity was in the ripe mesocarp. Activity in the separated zone was 3-5 times lower than in the mesocarp, and in tepal bases about 10 times lower than in the zone. The non-abscinding Kluang mutant zone-type cellulase exhibited an unusual pH profile, being most active at low pH, indicating a distinct difference from the cellulase induced in the normal separating ripe zone.

CHAPTER 6

PECTIC ENZYMES IN FRUIT TISSUES DURING RIPENING AND ABSCISSION - POLYGALACTURONASE AND PECTIN METHYLESTERASE

An initial examination of PG activity in unripe and ripe fruit tissues was determined with ammonium sulphate protein preparations (Section 2.4). Two protein fractions were obtained from each tissue. The first was the soluble protein extracted from the tissue with 20mM acetate buffer only. The second was the protein solubilised from the washed pellet (cell wall) of the first extraction with 250mM acetate/1M NaCl. The polygalacturonase activity in the different tissues is shown in Table 6.1. Abscission zone tissue which had separated had very high activity (an increase of about 30-fold compared with the unripe unseparated zone) but all other tissues had low activity.

TISSUE	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}			
	UNRIPE, NOT SEPARATED		RIPE, SEPARATED	
	Soluble	Cell Wall	Soluble	Cell Wall
Zone	4.2	1.2	125.7	1.3
Mesocarp	1.8	0.3	nd*	1.5
Tepal Bases	-	-	nd*	nd*
Pedicel	1.8	0.3	nd*	0.3

* nd = not detected

Table 6.1: PG Activity in Ammonium Sulphate Protein Preparations of Unripe Unseparated and Ripe Separated Fruit Tissues

Most of the enzyme activity in these preparations was solubilised in the first extraction with 20mM acetate buffer only. This indicated that the oil palm PG did not have strong ionic associations with the cell wall requiring desorption with 1M NaCl (unlike the tomato fruit PG). However, Knegt *et al*, (1988) reported that saturation to 80% with ammonium sulphate leads to appreciable losses in PG activity and thus, there may have been preferential loss in activity of the cell-wall bound enzyme. Therefore, the isolation of the "soluble" and "cell-wall" fractions by ammonium sulphate protein precipitation was discontinued. The next approach was to extract the tissue directly with and without NaCl. Since zone cells from separated ripe fruit contained the most PG

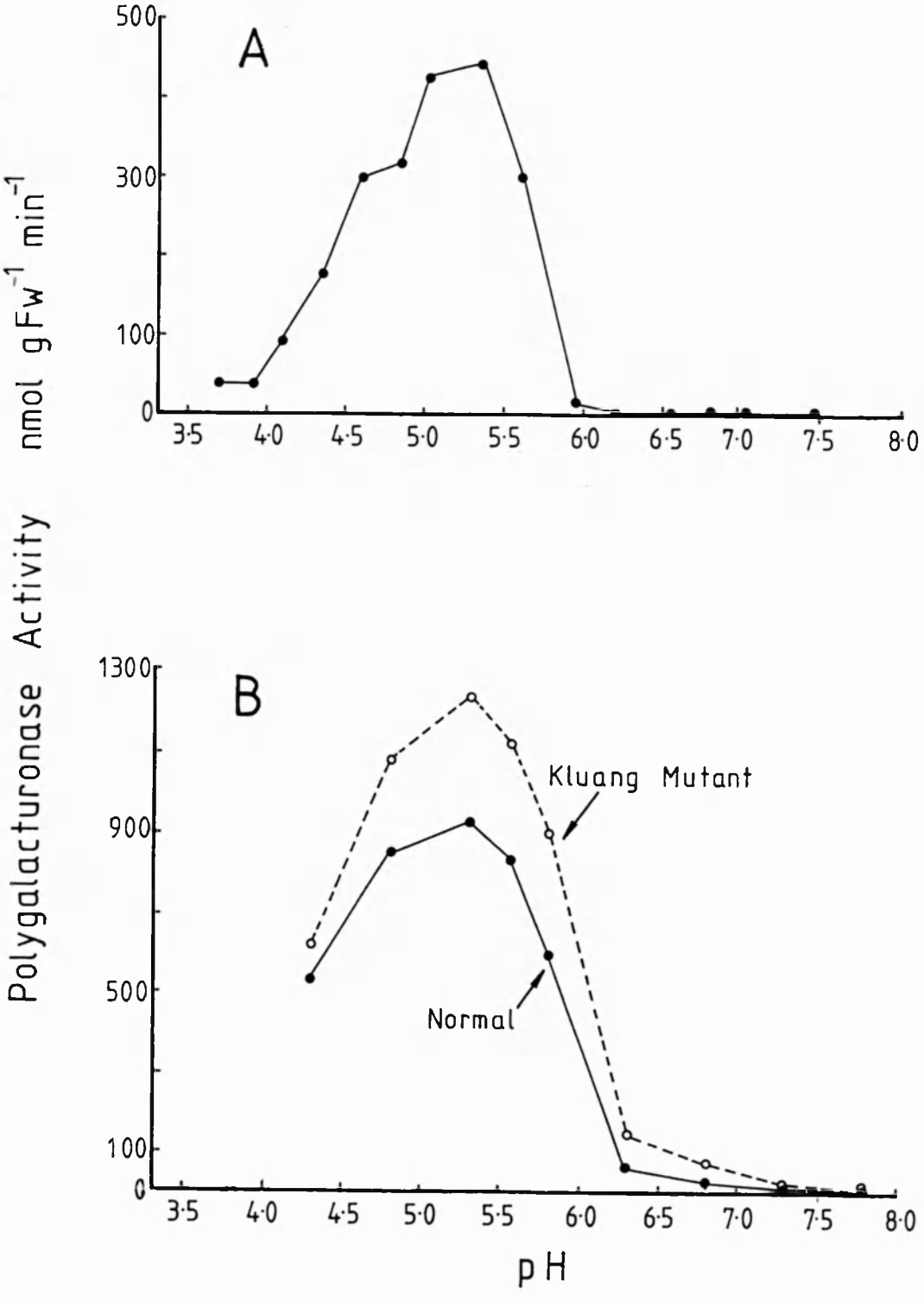


FIG. 6.1: THE PH-DEPENDENT POLYGALACTURONASE ACTIVITY

Enzyme extracts were prepared as described in Section 2.4.1 and PG activity was determined by the cyanoacetamide method for reducing groups (Gross, 1982).

A. The pH profile of normal ripe separated zone (150 daa) between pH 3.7-5.6 in 100mM acetate buffer and between pH 5.9-7.4 in 100mM phosphate buffer.

B. The pH profiles of ripe separated zone in both normal (152 daa) and the Kluang mutant (184 daa) between pH 4.3-5.8 in 100mM acetate and between pH 6.3-7.8 in 100mM phosphate.

activity, this tissue was used to determine whether NaCl in the extraction buffer was necessary. Zone tissue (FZ) of ripe fruit separated at 24h after arrival was sequentially extracted with water (x2) then with 250mM acetate buffer pH 5.0/1M NaCl (x2) and the PG activity is shown in Table 6.2.

Extractant	PG Activity nmol g Fw^{-1} min^{-1}
1. H_2O - first extraction	266.1
2. H_2O - extraction of pellet from (1) above	49.6
3. 250mM NaOAc/1M NaCl - Extraction of pellet from (2) above	157.9
4. 250mM NaOAc/1M NaCl - Extraction of pellet from (3) above	0.0

Table 6.2: PG Activity in a Separated Zone Sample Sequentially Extracted in Water (x2) and then 250mM Acetate pH 5.0 including 1M NaCl (x2)

In this comparison, 67% of the total PG activity was extracted with only the water, but a salt extraction did release further PG (33%) that was ionically bound to cell wall material. Thus, enzyme extracts for the assay of PG (and ß-1,3-GH) were extracted as described in Section 2.4.1, and desalted as described in Section 2.5.1.

In this chapter, PG activity is calculated as nmol reducing group g Fw^{-1} min^{-1}, that is, nanomoles reducing groups assayed by the cyanoacetamide method with galacturonic acid as a standard (as described in Section 2.6).

6.1 CHARACTERIZATION OF PG ACTIVITY IN NORMAL AND KLUANG MUTANT FRUIT

6.1.1 The pH-Dependent Polygalacturonase Activity

Separated zone (FZ/PZ) from ripe fruit (separated 1+2, 24h after arrival) was used for determining the pH optimum of the enzyme (extracted and assayed as described in Sections 2.4.1 and 2.6.2). Acetate and phosphate buffers (both at 100mM) were used for these determinations. The pH profile for extracts of tepal base 1 were the same as that for separated zone tissue (not shown). Figure 6.1A shows that, under the conditions of extraction and assay in 100mM acetate buffer, highest activity was obtained between pH 5.0-5.4. In this assay, PG was most active at pH 5.4. PG activity was negligible in the 100mM phosphate buffer at pH 5.9-7.4.

In order to determine if the pH optimum of PG was different in the Kluang non-abscinding mutant palm from the normal 271D clone, the pH-dependent activity of normal ripe zone (152 daa, separated on arrival) was compared with the Kluang mutant ripe zone (184 daa, the few fruit that had separated at Position 1 on arrival). Figure 6.1B shows that although the mutant zone sample had a little more activity than in the normal clone 271D, there was no difference in the pH profile.

6.1.2 Type of Substrate Hydrolysis: Exo and Endo

A decrease in the viscosity of a solution of polygalacturonic acid is usually a sensitive measure of endo-acting polygalacturonase activity. This method is particularly useful for measuring endohydrolase activity in the presence of exohydrolases since exohydrolase action over short periods does not lead to any appreciable change in the average molecular size of the substrate (Stone & Clarke, 1992). Although it was difficult to obtain a viscous solution of PGA, it was possible to determine that there was a decrease in viscosity by extracts of normal separated zone (FZ/PZ), but no activity was detected in extracts of other tissues by this method of assay. Viscosity experiments, with slight modifications, were repeated 8 times in all but only separated zone samples consistently showed a decrease in viscosity; extracts of unripe, unseparated zone and unripe and ripe mesocarp showed an **increase** in viscosity (Table 6.3).

	VISCOSITY CHANGE (17h)
Control, substrate and buffer	no change
Ripe separated zone, FZ/PZ	64.2 $\pm$ 9.8% decrease (n = 6)
Unripe unseparated zone	an increase in viscosity
Ripe and unripe mesocarp	an increase in viscosity

Table 6.3 **PG Activity in Enzyme Extracts of Unripe and Ripe, Zone and Mesocarp Tissues Measured by a Viscometric Assay**

The **increase** in viscosity observed in extracts other than separated zone must have been caused by unidentified gelling reactions of the PGA substrate with the enzyme extract and these reactions masked any viscosity decrease by low PG activity.

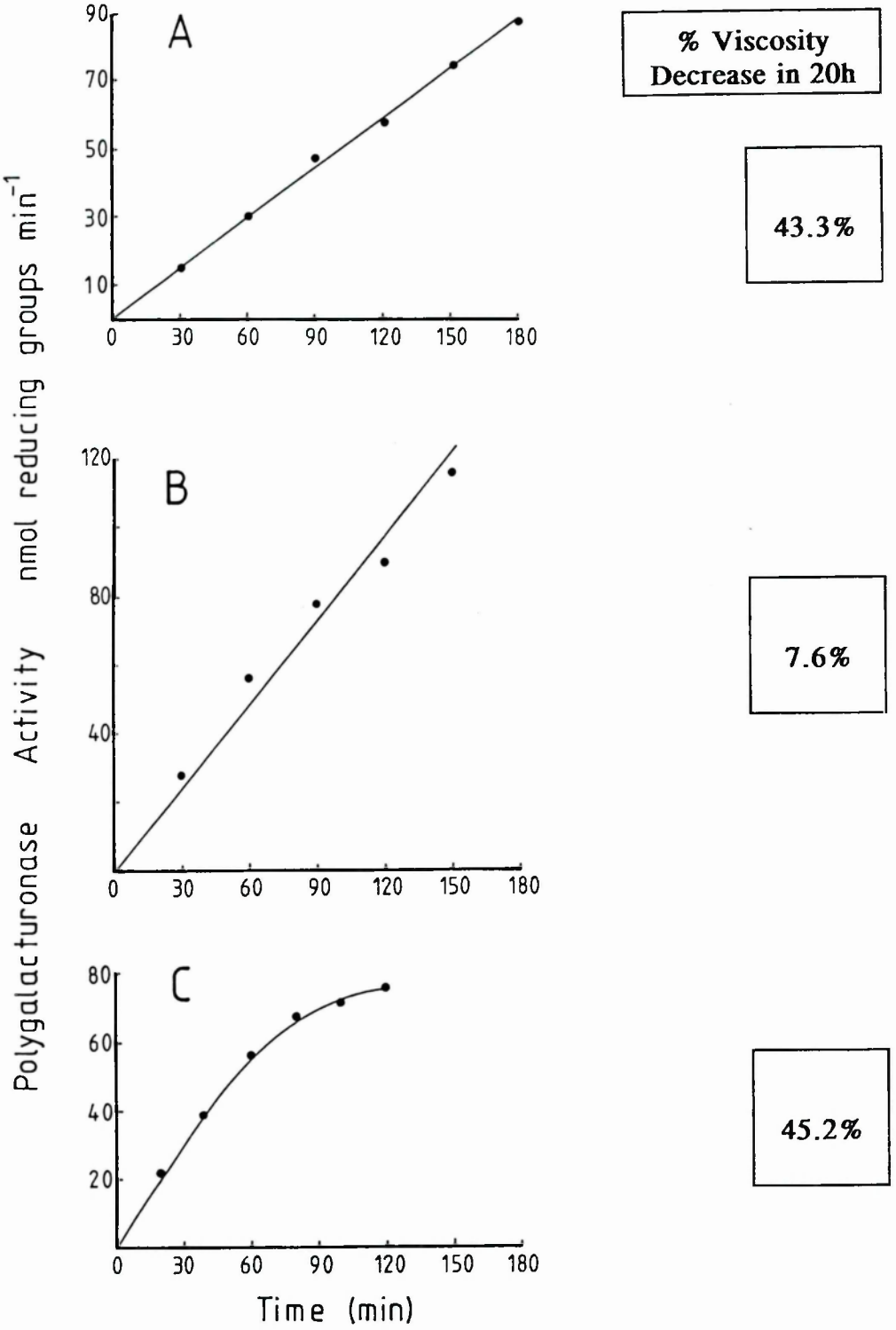


FIG. 6.2: TIME COURSE OF POLYGALACTURONASE ACTIVITY IN A TOTAL EXTRACT AND PARTIALLY PURIFIED FRACTIONS OF SEPARATED ZONE

A. The enzyme extract was prepared as described in Section 2.4.1. A time course of activity in the total extract was determined every 30min for 3h.

B. An extract of separated zone was fractionated by cation exchange chromatography. The fractions, eluted with 50mM NaCl, were pooled, concentrated, desalted and 50μl of the concentrate was assayed every 30min for 2.5h.

C. The 500mM NaCl eluted fraction from cation exchange chromatography, prepared as for (B) and assayed every 20min for 2h.

In addition, the viscosity change by a total extract of ripe separated zone was compared with the 50mM and 500mM NaCl-eluted fractions (pooled, concentrated and desalted) from fractionation by cation exchange chromatography (see Section 6.3.2). The PG activity in each concentrate was first measured by the cyanoacetamide reducing group assay and then an equal activity of each sample (~120 nmol reducing groups h^{-1}) was used for the viscosity assay (Table 6.4).

	% DECREASE IN VISCOSITY at pH 5.3	
RIPE SEPARATED ZONE Activity of each sample = 120 nmol reducing groups h^{-1}	**3h Incubation**	**20h Incubation**
Total extract concentrate	9.7	43.3
50mM NaCl eluted concentrate	0.0	7.6
500mM NaCl eluted concentrate	10.6	45.2

Table 6.4: PG Activity in Separated Zone Concentrates Measured by a Viscometric Assay.

6.1.3 Time Course of Polygalacturonase Activity

A substrate concentration of polygalacturonic acid of 10mg ml^{-1} maintained a linear rate of enzyme activity in total extracts of separated zone for up to 3h as shown in Fig. 6.2A. In other experiments this was observed for up to 6h. Thus, 10mg ml^{-1} substrate concentration was routinely used.

The PG isoenzymes fractionated on a cation exchange (Express-Ion C) column (with step-wise increases in NaCl concentration) (Section 6.3.2) also were tested for linearity of activity with time. The activity in fractions pooled from the 50mM NaCl elution (isoenzymes with pI 6.2-6.4) was linear and assumed to be exo-acting (Fig. 6.2B). The activity in fractions pooled from the 500mM NaCl elution (pI >8.5) became substrate limited by 2h and suggests that this enzyme is endo-acting (Fig. 6.2C). PG activity in fractions from the 100mM NaCl elution, after concentration and electrophoresis on an IEF gel, activity and silver staining indicated that a small amount of the pI 6.4 isoenzyme may be present (Fig. 6.9B) in addition to the isoforms with pI 8.5. Thus, an enzyme assay of this fraction might then represent the combined activities of the endo- and exo-enzymes.

6.1.4 The Stability of Polygalacturonase Activity

Oil palm tissue was a valuable commodity because, not only was the shipment from Malaysia to Oxford expensive, but fruit from the few mutant palms on the plantations was very limited. Therefore, it was necessary to know whether stored frozen tissue (-20°C) lost activity and over what period of time it could be used without loss. Tests over 14 months showed the enzyme to be remarkably stable.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
SEPARATED ZONE, FZ	**First Extract of Tissue on Arrival**	**Tissue Extracted 14 Months Later**
Normal fruit (155 daa)	586.7	568.2
Kluang mutant fruit (184 daa)	870.0	857.5

Table 6.5: PG Activity in Separated Zone Extracts on Arrival and in Stored Frozen Tissue after 14 Months

In addition, zone tissue (scraped cells) was unfrozen and left for 24-120h (1-5 days) at 10°C or 24-72h at 23°C, and then re-frozen. Although this caused the tissue to brown, the activity of polygalacturonase subsequently extracted was essentially unchanged.

SEPARATED ZONE, FZ	PG ACTIVITY nmol g Fw^{-1} min^{-1}
Control tissue, frozen	531.7
Tissue at 10°C for 24h	564.0
Tissue at 10°C for 72h	554.8
Tissue at 10°C for 120h	513.2
Tissue at 23°C for 24h	736.2
Tissue at 23°C for 72h	683.1

Table 6.6: PG Activity in Enzyme Extracts of Separated Zone Tissue (148 daa), Thawed for 0, 1, 3 and 5 days at 10°C; 1 and 3 days at 23°C.

These experiments showed that the polygalacturonase enzymes did not lose their activity when zone tissue was removed from the fruit. In fact, separated zone tissue left at room temperature for 24-72h (1-3 days) appeared to have about 30% more activity. Since PG readily adheres to the pectins, the increase observed may have been due to hydrolysis of pectins/cell wall material resulting in more readily extractable PG enzyme.

The effect of freezing (-20°C) and then unfreezing the same preparation was investigated since, at times, it was necessary to prepare extracts prior to analysis. Polygalacturonase activity was measured in separated zone tissue of the normal and mutant fruit and as the results show, there was no loss of activity if the extracts were frozen.

	POLYGALACTURONASE ACTIVITY OF ENZYME EXTRACT nmol g Fw^{-1} min^{-1}				
Separated Zone, FZ	**Before Freezing**	**Unfrozen**			
		x 1	**x 2**	**x 3**	**x 4**
Normal (148 daa)	711.1	638.6	-	666.4	635.2
Kluang Mutant (184 daa)	857.4	-	886.6	823.9	873.9

Table 6.7: PG Activity in Separated Zone Immediately After Preparation of Enzyme Extracts and then After Freezing and Thawing the Same Extract

Thus, it was possible to collect tissue samples, freeze them, and then later extract, assay and re-assay without significant losses in the PG activity.

6.1.5 The Effect of Type of Buffer and Other Substances on PG Activity

Methods of extraction and assay of polygalacturonase in ripening fruit are well documented and include the use of citrate, phosphate or Tris buffers and EDTA. The effects of these conditions were studied on the oil palm abscission zone PG. However, these substances had an inhibitory effect on PG. The activity of PG in 100mM phosphate buffer at pH 5.8 was greatly reduced compared with 100mM acetate buffer at the same pH (6% cf 43% of the maximum) indicating an inhibitory effect of the phosphate ion. Citrate buffer (100mM) was also inhibitory (24% of the maximum). Tris/HCl-Tris/glycine buffers are routinely used in anionic native electrophoresis (Davis, 1964). The PG activity at high pH (pH 8.6-8.9) was negligible (10-16% of the maximum) and was not restored when the gels were re-equilibrated to pH 5.3. These results are summarised in Table 6.8.

Assay Conditions	PG ACTIVITY IN SEPARATED ZONE (FZ)	
	nmol reducing groups g Fw^{-1} min^{-1}	% PG Optimum Activity in 100mM Acetate pH 5.3
100 mM Acetate pH 5.0	420.6	98%
100 mM Acetate pH 5.3	430.4	100%
100 mM Acetate pH 5.5	347.5	81%
100 mM Acetate pH 5.8	185.6	43%
100mM Phosphate pH 5.8	23.9	6%
100mM Citrate pH 5.6	94.2	24%
Tris/Glycine pH 8.6	44.9	10%
Tris/HCl pH 8.9	68.9	16%

Table 6.8: PG Activity in Acetate, Phosphate, Citrate and Tris Buffers at Different pHs.

Ampholytes used in electrophoresis were also examined for their effect on PG activity. Those in the IEF gels were highly inhibitory. For example, this was tested on an extract of ripe separated zone with a PG activity assayed as 38.0nmol/h. However, with 1.25% ampholyte pI 5-7 included in the assay, the activity was reduced to 4.1nmol/h (~11% of the original activity) but with 0.5% ampholyte in the assay, activity was unaffected. Ampholyte concentration in the Ampholine PAGplate is 2.2%. Therefore, removal of ampholytes is essential in order to obtain an active polygalacturonase in the gel.

In assays (1h) of separated zone, EDTA (10mM) completely inhibited PG activity but when more dilute samples were assayed for a longer time (18h) only 52% inhibition was obtained (Table 6.9). The retardation of separation in the fruit when treated with EDTA (Section 3.5.1) could, therefore, be due to the inhibition of PG.

SEPARATED ZONE FZ/PZ	nmol reducing groups produced in 18h	% PG Activity
Control (n = 3)	74.3 ± 23.5	100%
10mM EDTA (n = 3)	39.1 ± 12.9	52%

Table 6.9: The Effect of EDTA on PG Activity in Extracts of Separated Zone Tissue.

Since citrate (a tricarboxylic acid) and EDTA (four carboxylic acid groups) were inhibitory to PG, the effect of some other dicarboxylic acids on PG activity was examined. They, too, all had an inhibitory effect (Table 6.10).

Assay Conditions, pH 5.3	PG ACTIVITY IN SEPARATED ZONE (FZ/PZ)	
	nmol reducing groups g Fw^{-1} min^{-1}	% PG Optimum Activity in 100mM Acetate pH 5.3
100 mM Acetate	639.9	100.0%
100 mM Oxalate	153.1	23.9%
100 mM Malonate	227.9	35.6%
100 mM Malate	244.3	38.2%
100mM α-ketoglutarate	144.7	22.6%

Table 6.10: **The Effect of Some Dicarboxylic Acids on PG Activity in Extracts of Separated Zone Tissue**

Inclusion of galacturonic acid (25nmol in assay) or digalacturonic acid (12.5nmol in assay) did not alter PG activity, indicating that end-product inhibition was unlikely.

6.2 PG ACTIVITY IN THE DIFFERENT TISSUES OF NORMAL AND KLUANG MUTANT FRUIT

6.2.1 Comparison of PG Activity in Tissues of the Normal Clones 271D, 90A and 476G

Three normal clones, 271D, 90A and 476G, were compared. All the fruit were fully ripe and separated either on arrival or within 24h.

RIPE TISSUE	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}		
	271D	90A	476G
Separated Zone FZ/PZ	190.3	159.8	191.6
Fruit Periphery	59.2	49.2	36.3
Mesocarp	18.3	19.6	23.9
Tepal Base 1	1.3	3.3	2.3
Tepal Base 2	1.9	-	2.8

Table 6.11: **PG Activity in Enzyme Extracts of the Different Tissues in Separated Ripe Fruit of Three Normal Clones, 271D, 90A and 476G.**

This experiment showed that the activity of PG in the different tissues of other clones followed the same pattern of distribution, with highest activity in the separated abscission zone. (However, higher activities for separated zone and tepal bases were obtained in subsequent experiments due to an improved extraction procedure; Section 2.4.1).

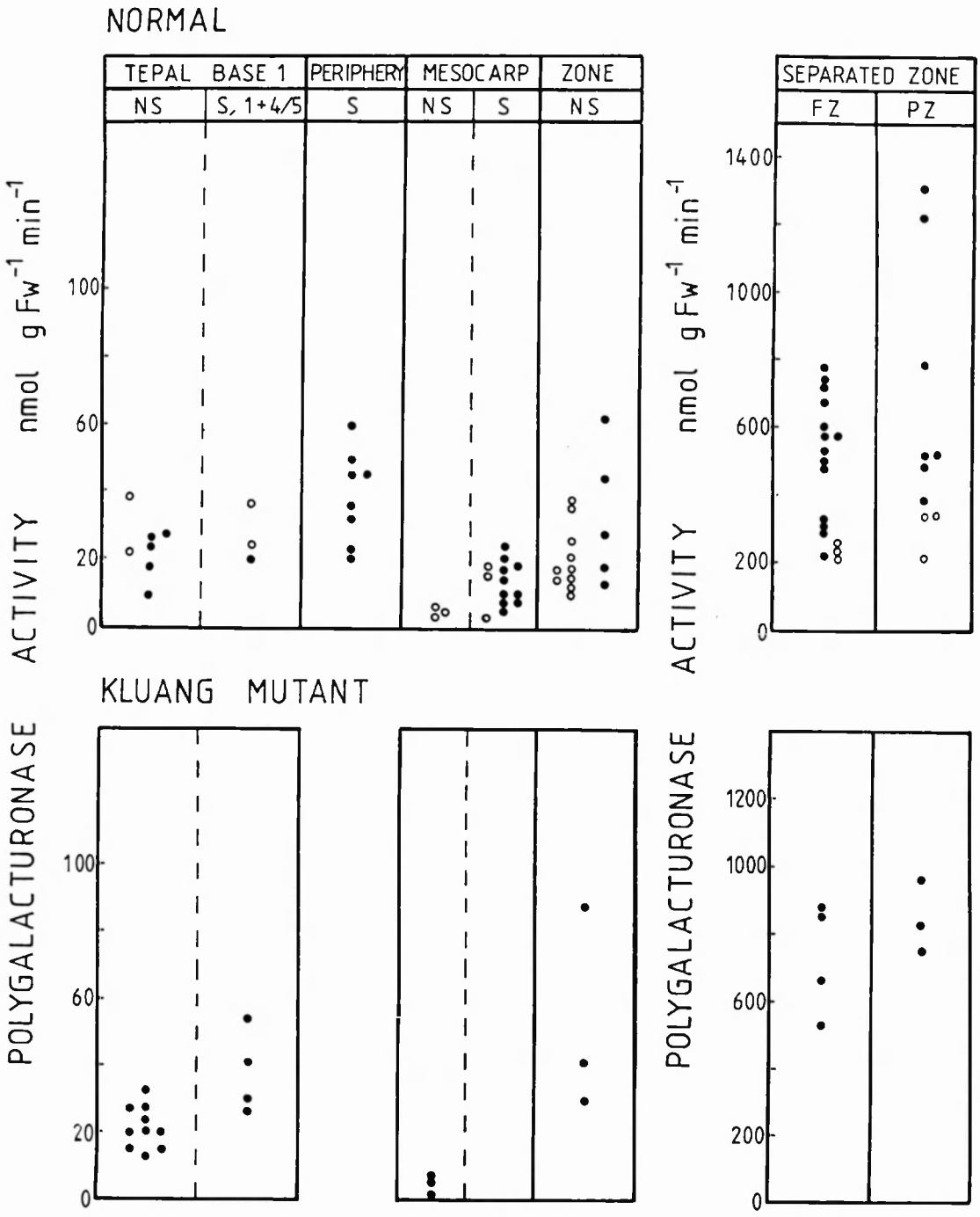


FIG. 6.3: THE POLYGALACTURONASE ACTIVITY IN THE DIFFERENT TISSUES OF NORMAL AND KLUANG MUTANT FRUIT

The enzyme extracts were prepared at the same time and assayed (reducing groups) to compare the activity in unseparated and separated tissues of both the normal and Kluang mutant fruit.
(Note that the scale for the separated zone is x10.)

○ Ripening ● Ripe

6.2.2 Comparison of PG Activity in Normal and Kluang Mutant Fruit Tissues

Extracts of the different tissues of normal and mutant fruit were all prepared at the same time in the same buffer stock for extraction, desalting and assay, so that the PG activity could be directly compared.

- **The Unseparated Zone**

PG activity in the unseparated zone (even of ripe fruit) was always low. The actual PG activity of the unseparated zone may not be exactly as in this assessment since any unseparated zone slice when excised from longitudinal sections of the fruit must include mesocarp tissue above and pedicel below in addition to the zone tissue. Some mesocarp and pedicel tissue must therefore contribute to the total PG value obtained.

Table 6.12 compares the activity in the unseparated zone of normal and Kluang mutant fruit (mean ± standard deviation, number of different samples tested, and the range of activity) on arrival in the laboratory. There was slightly higher activity in the mutant and this may have been due to the slower progression of the separation. The individual results are shown in Fig. 6.3.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
UNSEPARATED ZONE	**RIPENING**	**RIPE**
NORMAL	**20.7 ± 9.5** n = 10 (12.8-37.5)	**32.9 ± 20.5** n = 5 (12.7-62.2)
KLUANG MUTANT	-	**56.2 ± 27.1** n = 3 (39.7-87.5)

Table 6.12: PG Activity in the Unseparated Zones of Ripening and Ripe Normal and Kluang Mutant Fruit.

The high activity in two of the ripe normal samples (44.7 nmol g Fw^{-1} min^{-1} and 62.2 nmol g Fw^{-1} min^{-1}) corresponded with the onset of translucency in the unseparated zones (which preceded cell separation). Some fruit of the 62 nmol g Fw^{-1} min^{-1} sample were still attached at the fruit periphery after 48h (separation at Position 1 only). Therefore, although it was possible to excise this zone, it still had adhering mesocarp and pedicel tissue so that at this stage it was comparable to the unseparated zone tissue. The

PG activity increased from a maxiumum of 62.2 nmol g Fw^{-1} min^{-1} to a minimum of 272.7 nmol g Fw^{-1} min^{-1} on separation, which is a 4-fold increase from the translucent stage to separation at Position 1 (Fig. 6.3).

- **The Separated Zone FZ/PZ**

The separated zone tissue consists of pooled cells scraped from the fruit side (FZ) and pedicel side (PZ) of a number of fruit. Previous experiments had indicated that, on a per gram fresh weight basis, PG activity was higher in the pedicel side (PZ) of the zone than the fruit side (FZ). An interesting trend was observed in fruit which were at the various stages of abscission on arrival, and those which separated 24h or more after arrival.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
STAGE OF SEPARATION	**FRUIT SIDE (FZ)**	**PEDICEL SIDE (PZ)**
SEPARATED ON ARRIVAL:		
Fruit shed in transit	586.7	1224.8
Fruit shed in transit	571.7	1304.2
Fruit off easily with slight pressure	281.7	483.3
Fruit off easily with slight pressure	215.0	381.7
Fruit off with pressure, snapping off	198.3	138.3
NOT SEPARATED ON ARRIVAL BUT:		
Ripe fruit separated 24h after arrival	771.8	512.7
Ripe fruit separated 48h after arrival	332.0	783.5
Ripening fruit separated 48h after arrival	207.5	219.3
Ripening fruit separated 48h after arrival	264.2	343.2
Ripening fruit separated 72h after arrival	234.0	341.2

Table 6.13: PG Activity in Extracts of Separated Zone Samples, on Arrival in the Laboratory and 24h or More After Arrival.

The results indicate that the extent of the increase in PG activity on the pedicel side of the zone may be important in determining the natural separation of the fruit. In fact, observation of the region of translucency shows that the separation occurs closer to the pedicel side (Fig. 3.6, page 82A). The PG activity in FZ and PZ when both ripe and unripe fruit separate after the spikelets have been harvested never reached the high levels

obtained from fruit shed in transit (that is, fully ripe and near to shedding on the plantation).

Table 6.13 also shows that the variation in PG activity in the separated zone can be related to the nearness to abscission. That is, from fruit shed in transit or those which separate easily with no resistance (>500 nmol g Fw^{-1} min^{-1}), to their being gently pressed off (>200 nmol g Fw^{-1} min^{-1}) and finally to when they require some pressure to be removed (snapped off, Position 1 only) (<200 nmol g Fw^{-1} min^{-1}). In addition, ethylene production by fruit shed in transit or on arrival was higher than that from fruit which had separated at Position 1 only (Fig. 2.3, page 46A and Section 4.2.1, page 101). Thus, the increased PG activity could be due to the increased ethylene production which induces greater synthesis of PG enzyme.

The PG activity in FZ and PZ tissue of the Kluang mutant fruit was also examined. Since very few Kluang mutant fruit ever separate in the field (and hence very few were separated on arrival), it was not possible to obtain the naturally shed values. However, the samples analysed show that the levels of PG activity were similar to those of normal fruit when the mutant separates 24h or more after arrival in the laboratory.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
STAGE OF SEPARATION	**FRUIT SIDE (FZ)**	**PEDICEL SIDE (PZ)**
184 daa separated on arrival (with snapping)	870.0	962.0
184 daa separated 24h after arrival (with snapping)	538.0	825.3
218 daa separated 48h after arrival	666.3	750.3

Table 6.14: PG Activity in Extracts of Separated Zone Tissue, FZ and PZ, in Kluang Mutant Fruit

The PG activity of normal and Kluang mutant separated abscission zones, FZ and PZ, is compared in Table 6.15 (mean $\pm$ standard deviation, number of different samples analysed, and the range), with the individual results shown in Fig. 6.3.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
SEPARATED ZONE	**RIPE FZ**	**RIPE PZ**
NORMAL (147 daa - 157 daa)	**518.8 ± 179.0** n = 14 (215.0-771.8)	**743.1 ± 377.1** n = 7 (381.7-1304.2)
KLUANG MUTANT (184 daa - 218 daa)	**733.0 ± 160.0** n = 4 (538.0-870.0)	**845.9 ± 107.3** n = 3 (750.3-962.0)

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
SEPARATED ZONE	**RIPENING FZ**	**RIPENING PZ**
NORMAL (124 daa - 139 daa)	**235.2 ± 28.4** n = 3 (207.5-264.2)	**301.2 ± 71.0** n = 3 (219.3-343.2)
KLUANG MUTANT	-	-

Table 6.15: PG Activity in Extracts of Separated Zone, FZ and PZ, in Ripe Normal and Kluang Mutant Fruit compared with Ripening Normal Fruit

It is clear that PG activity increased as separation occurred (compare Tables 6.12 and 6.15) but although separation was slower in the Kluang mutant fruit, the levels of PG activity attained overlap with those in normal fruit. Although the Kluang mutant separated FZ and PZ samples analysed showed a mean PG activity of ~40% and 14% higher respectively than the normal, this may have been due only to their slower separation and continued synthesis of PG enzyme in the presence of high ethylene production rates. *A priori,* there seems no reason to associate the lack of abscission with the failure of PG induction.

- **The Fruit Periphery**

The periphery represents the outer face of the fruit base, and **before** separation it is attached to tepal base 1, the area immediately below the RA, that is, the tissue between Positions 2 and 3 (Fig. 2.1, page 44A). The tissue is difficult to dissect without some adhering mesocarp tissue. However, the PG activity is higher here (38.7 ± 13.4 nmol g Fw^{-1} min^{-1}) than in the ripe mesocarp (13.5 ± 5.9 nmol g Fw^{-1} min^{-1}). Therefore, PG may be involved in the final separation of the base of the fruit from tepal base 1 when

separation at Position 2/3 is not by-passed. The individual values are shown in Fig. 6.3.

- **Mesocarp and Pedicel Tissue**

Mesocarp PG activity in ripening and ripe fruit was always low in both the normal and the Kluang mutant fruit as shown in Table 6.16.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
MESOCARP	**RIPENING**	**RIPE**
NORMAL Unseparated fruit	**5.0 ± 1.5** n = 3 (3.7-6.7)	-
Separated fruit	**12.8 ± 8.1** n = 3 (3.5-18.7)	**13.5 ± 5.9** n = 10 (6.5-23.9)
KLUANG MUTANT Separated fruit	-	**4.3 ± 2.6** n = 3 (1.3-6.3)

Table 6.16: PG Activity in Enzyme Extracts of Ripening and Ripe Mesocarp from Normal Unseparated and Separated Fruit and from Kluang Mutant Separated Fruit.

PG activity in the mesocarp was essentially unchanged as ripening progressed, remaining low in both ripening and fully ripe tissue and 50-100 times lower than in separated zone.

In the pedicel tissue just below the PZ (48h after arrival), PG activity was similar to that found in the mesocarp (21.7 ± 8.7 nmol g Fw^{-1} min^{-1}, n = 3).

The PG activity of unseparated, ripe normal zone (which was excised and comprises mesocarp and pedicel tissue) was 32.9 ± 20.5 nmol g Fw^{-1} min^{-1} and of the Kluang mutant, 56.2 ± 27.1 nmol g Fw^{-1} min^{-1}. Thus, since the PG activity in mesocarp and pedicel was only slightly lower than these values, this indicates that the level of PG activity in the unseparated zone tissue of both normal and mutant fruit was also low.

- **Rudimentary Androecium**

When the rudimentary androecium in ripe fruit (separated 1+3) was assayed separately from the tepal base 1, the RA itself was found to have very little PG activity.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
TISSUE	**Separated 1+3 after 24h**	**Separated 1+2/3 after 48h**
Rudimentary androecium	0.3	1.0
Tepal base 1	5.3	8.0

Table 6.17: **PG Activity in Enzyme Extracts of the Rudimentary Androecium and Tepal Base 1.**

- **Tepal Base 1**

When ripe fruit shed naturally, 1+2/3 (in transit or on arrival in the laboratory), the tepals are still attached to the pedicel (Fig. 5.5B, page 119A). If the inner tepal 1 is removed at this time (with force), PG activity was high on the tepal base and similar to levels in the fruit side of the zone ("fruit shed in transit" in Table 6.18, compare with FZ in Table 6.13), presumably due to adhering zone tissue.

In contrast, in ripe fruit which are not close to natural abscission, the unseparated tepal base 1 had low PG activity (21.2 $\pm$ 7.3 nmol g Fw^{-1} min^{-1}). Even when this whorl of tepals separated from the pedicel (but attached to the fruit, 1+4/5 separation, Fig. 5.5D, page 119A), PG activity in the tepal base still remained low (19.3 nmol g Fw^{-1} min^{-1}). Similar levels of PG activity were obtained in tepal base 1 of ripening fruit (Table 6.18).

TEPAL BASE 1	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}
TEPALS ATTACHED TO PEDICEL	
Ripe fruit shed 1+2 in transit (155 daa, n = 1)	**576.0**
Ripe fruit shed 1+2 in transit (152 daa, n = 1)	**170.3**
Ripe fruit not shed, or unseparated at Position 2/3	**21.2 ± 7.3** n = 5 (9.5-27.0)
Ripening unseparated fruit	**30.3 ± 11.7** n = 2 (22.0-38.5)
TEPALS SEPARATED FROM PEDICEL	
Ripe fruit separated 1+4/5	**19.3** n = 1
Ripening fruit separated 1+4/5	**30.2 ± 9.2** n = 2 (23.7-36.7)

Table 6.18: PG Activity in Enzyme Extracts of Tepal Base 1 Attached to or Separated from the Pedicel in Ripening and Ripe Normal Fruit.

The Kluang mutant essentially does not have fruit which separate naturally 1+2, and of the many fruit received only 2 fruit were ever observed to separate 1+2 with no resistance. Thus, tepal base 1 samples collected from these mutant fruit were always attached to both fruit and pedicel (Fig. 5.5A, page 119A). When the mutant fruit separated in the laboratory, tepal base 1 had separated from the pedicel but remained attached to the fruit (1+4/5 separation, Fig. 5.5D, page 119A). The PG activity of these tepal base 1 samples was similar to that in normal fruit. The results are shown in Table 6.19.

TEPAL BASE 1	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}
KLUANG MUTANT (Ripe) Unseparated from pedicel	21.3 ± 6.1 n = 10 (13.3-32.5)
Separated from pedicel (1+4/5)	38.2 ± 12.5 n = 4 (26.7-54.5)

Table 6.19: PG Activity in Enzyme Extracts of Tepal Base 1 Attached To or Separated From the Pedicel in Ripe Kluang Mutant Fruit.

All these results suggest that although PG activity at Position 2/3 is necessary for natural fruit separation (1+2/3), PG is not induced when tepals separate from the pedicel (4/5 separation). Whether the tepal is attached to the pedicel or is separated from it, the PG activity is low. In fact, the PG activity of tepal base 1 was similar to that found in pedicel tissue (21.7 ± 8.7 nmol g Fw^{-1} min^{-1}).

In addition to the above experiments, in which tepal base 1 samples were extracted and assayed at the same time, many previous experiments confirmed that PG activity was always low in tepal base 1: less than 10 nmol g Fw^{-1} min^{-1} (4.2 ± 3.4 nmol g Fw^{-1} min^{-1}, n = 29), and no differences were observed between ripening or ripe, unseparated or separated.

- **Tepal Base 2**

Tepal base 2 has no attachment to the fruit, and after the two whorls of tepals have separated from the pedicel (Fig. 2.4D, page 47A) tepal base 2 tissue comprises Position 5 only. In normal ripe separated tepal base 2, the PG activity was 25.2 ± 4.5 nmol g Fw^{-1} min^{-1} (n = 2), and was similar to the activity found in tepal base 1.

6.2.3 The Effect of Applied Ethylene on PG Activity in Normal Fruit

Ripening normal fruit on arrival do not produce ethylene. To test the effect of applied ethylene, spikelets (139 daa) were divided and placed into glass tanks (12 litres)

with lids for 48h. One tank contained air with an open dish of mercuric perchlorate to absorb ethylene, and the other tank was injected with ethylene (through a Suba-seal stopper inserted into the lid) to give 10μl l^{-1}. The tanks were aerated after 24h, after which the ethylene tank was re-injected. After 48h, all the fruit on the spikelets in the ethylene tank separated easily but fruit on spikelets in the air tank were pressed off with some force and some could **not** be separated. Separated zone FZ/PZ, mesocarp and tepal base 1 tissue samples were collected and analysed for PG activity. PG activity was higher, especially in the zone (~40%) when fruit were held in ethylene.

	POLYGALACTURONASE ACTIVITY nmol g Fw^{-1} min^{-1}	
RIPENING (139 daa)	**Air + M.P.**	**Ethylene**
Separated zone FZ/PZ	178.2	252.2
Mesocarp	16.1	18.7
Tepal Base 1	1.4	3.7

Table 6.20: PG Activity in Enzyme Extracts of Ripening Fruit Tissues after Incubation (48h) in either Air + MP or Ethylene.

6.2.4 PG Activity and Ethylene Production Compared in Normal and Kluang Mutant Fruit

Enhanced separation could be induced when the unripe and ripening Kluang mutant fruit were held in ethylene (10μl l^{-1}), or when the fruit were treated with a solution of ACC (1mM) (Section 4.2.2, page 102). However, although the ripe untreated Kluang mutant fruit are producing ethylene at levels which would result in PG induction and fruit separation in normal fruit, the Kluang mutant does **not** separate. The PG activity in the normal and Kluang mutant unseparated zone tissue was essentially similar, although the ethylene production in the mutant fruit was orders of magnitude higher than in the normal fruit, as shown in Table 6.21.

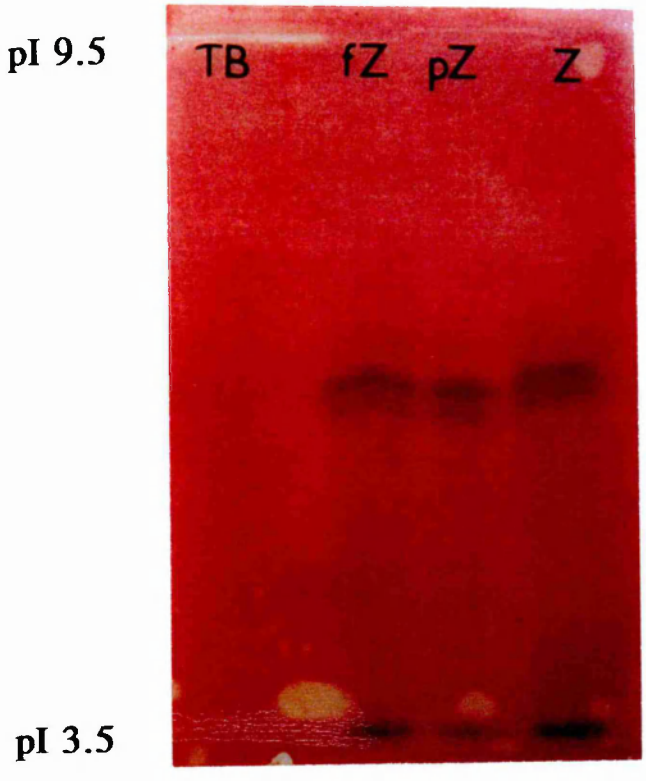


FIG. 6.4: THE POLYGALACTURONASE ISOENZYMES IN SEPARATED ZONE TISSUE

Enzyme extracts of tepal base 1, FZ, PZ and FZ/PZ combined were fractionated by isoelectric focusing. After incubation with an overlay of PGA substrate (2h), the gel was stained with TTC as described in Section 2.7.1. The approximate pI of 6.4-6.2 was determined by comparison with a silver-stained gel of an IEF standard protein mix (see Figs. 2.8, 6.5 and 7.3).

The "band" at pI 3.5 is negatively-charged reducing groups present in the enzyme extract, possibly uronic acids (see Fig. 2.7).

UNSEPARATED ZONE TISSUE	PG ACTIVITY nmol g Fw^{-1} min^{-1}	C_2H_4 PRODUCED nl g Fw^{-1} h^{-1}
KLUANG MUTANT		
184 daa terminal fruit on arrival	87.5	1.4 ± 0.5 (n = 6)
167 daa, 24h after arrival	39.7	-
194 daa, 120h after arrival	41.3	1.1 ± 0.6 (n = 3)
NORMAL		
Ripe (144-145 daa; n = 5)	32.9 ± 20.5	<0.01 - 0.01
Ripening (115-133 daa; n = 10)	20.7 ± 9.5	0.0

Table 6.21: PG Activity in Enzyme Extracts of Unseparated Zone and the Ethylene Production Compared in Ripe Kluang Mutant Fruit with Ripening and Ripe Normal Fruit.

These results in the Kluang mutant fruit suggest that ethylene **alone** is not the regulator of PG activity and that cell wall dissolution at separation is dependent upon the activity of other cell wall glycohydrolases which must act prior to or in concert with PG.

6.3 THE ISOENZYMES OF PG IN NORMAL AND KLUANG MUTANT FRUIT

Enzyme extracts of normal separated zone FZ, PZ, and FZ/PZ together, and tepal base were prepared for gel electrophoresis (Section 2.7). They were then fractionated on IEF gels and stained for activity with TTC (Section 2.7.1). Two bands of activity are observed in separated zone (Fig. 6.4) with no difference between FZ or PZ, but in the tepal base no bands of PG activity are visualised.

The Kluang mutant zone, which is slow to separate in the laboratory, was then compared with the normal zone. Enzyme extracts of the normal and the Kluang mutant fruit were prepared for gel electrophoresis by using the same fresh weight and concentrating to the same volume. Pectin in the extracts was removed by filtration through a 100K ultrafilter; if pectin was not removed, the proteins did not focus well.

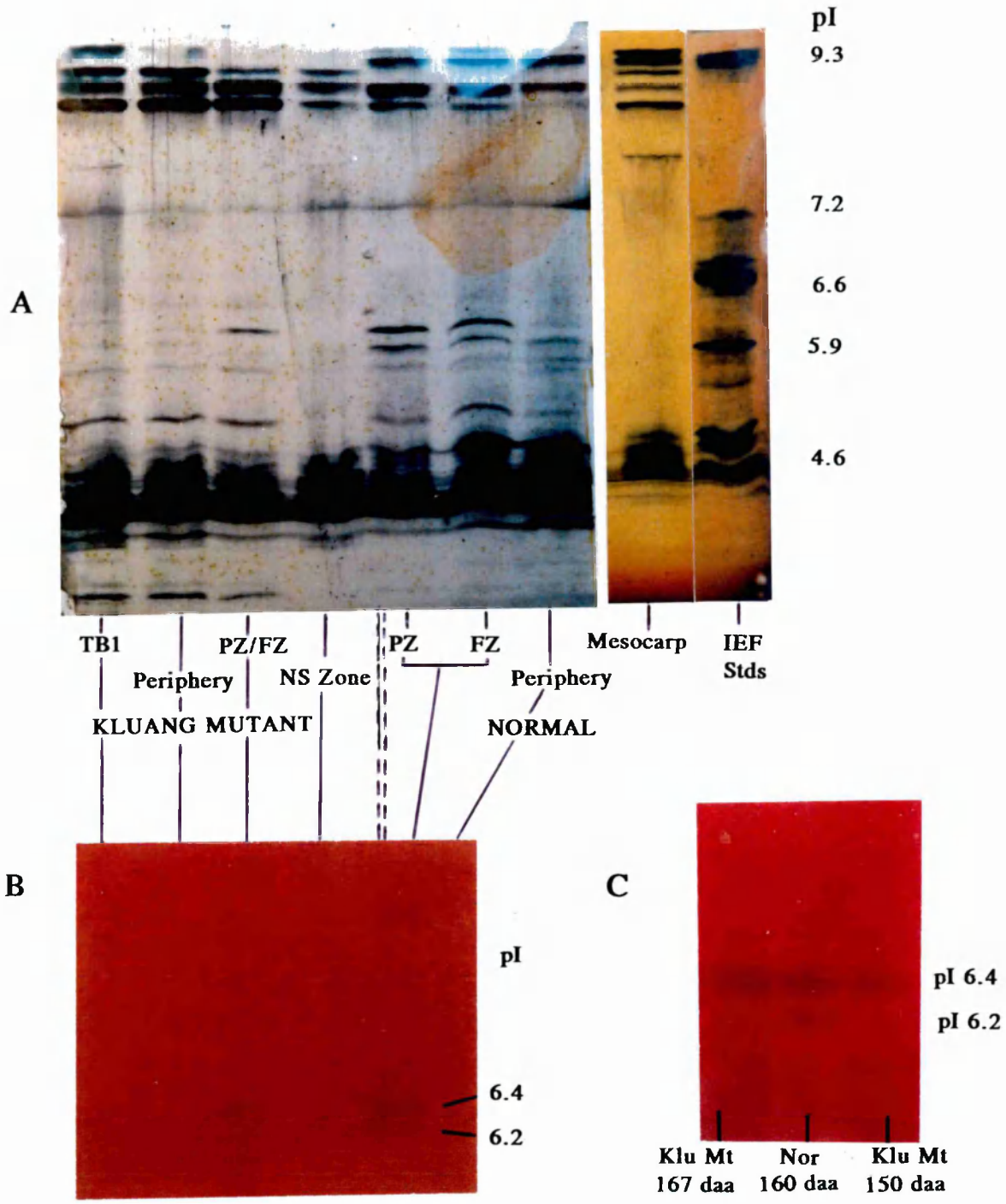


FIG. 6.5: THE POLYGALACTURONASE ISOENZYMES (PI 6.2-6.4) IN NORMAL AND KLUANG MUTANT FRUIT

Enzyme extracts of fruit tissues (concentrated by ultrafiltration) were fractionated by isoelectric focusing (pI 3.5-9.5), and the pI 6.2-6.4 isoenzymes compared.

A. IEF gels silver-stained for protein.

B. IEF gels of the same extracts in (A) stained for PG activity with TTC.

C. Extracts of the Kluang mutant zone (167 daa and 150 daa) which separated 120h after arrival in the laboratory, and an extract of normal separated zone (160 daa) were fractionated by isoelectric focusing (pI 5.5-8.5) and stained for PG activity with TTC.

All the extracts were then concentrated in a 3K ultrafilter before applying to the same gel and fractionated by isoelectric focusing. The gel was then cut, silver-stained for protein (Fig. 6.5A) and stained for PG activity with TTC (Fig. 6.5B). Figure 6.5 shows photographs of the gels of the normal and Kluang mutant tissue extracts from separated and unseparated zone, fruit periphery, tepal base and mesocarp.

The most noticeable difference was that the Kluang mutant separated zone appeared to have only one of the two bands of PG observed in normal FZ/PZ (Fig. 6.5A). Initially, it was thought that the absence of this isoenzyme may have been a reason why the fruit were non-abscinding in the field and slow to separate after harvesting. However, further extracts of the Kluang mutant separated zone (after isoelectric focusing and staining for activity) indicated that there was some activity at pI 6.2 (Fig. 6.5C), although it was always less than that observed in the normal. No PG activity on the IEF gels was detected with TTC for mesocarp, tepal base, unseparated zone, or periphery. Because concentration of the enzyme extracts by ultrafiltration did not completely remove the pectins and, in order to resolve the differences between the mutant and normal zone PG isoenzyme patterns, extracts were subjected to further purification.

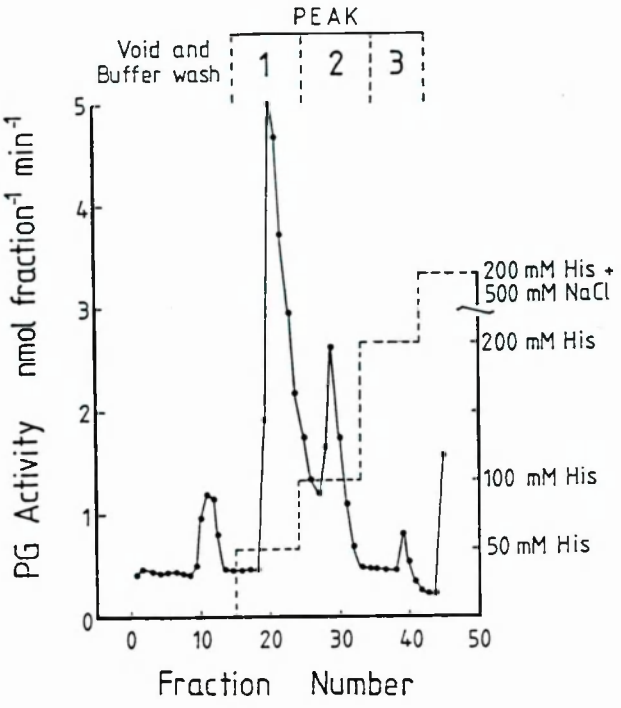
In addition, whilst the TTC reagent is particularly useful in the detection of monosaccharide reducing sugars (Gabriel & Wang, 1969), the extent of its reaction with reducing sugars in oligo- or polysaccharides is not known. For this reason, the two bands of activity visualised with TTC suggested a likely exo-PG activity. However, viscometric assays of separated zone had indicated that there was certainly an endo-PG activity present.

To determine which isoenzymes of PG were present in the different oil palm fruit tissues, extracts were purified as follows. Initially, anion exchange chromatography was used to remove the pectin from extracts and investigate further the apparent lack in the mutant zone of one of the two PG isoenzymes visualised in normal separated zone.

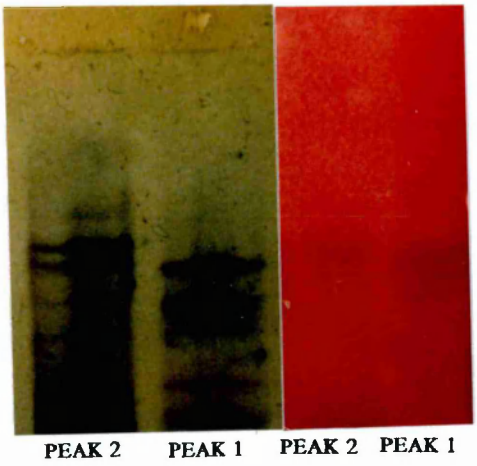
6.3.1 The pI 6.2-6.4 Isoenzymes

A sample of the normal separated zone in 20mM triethanolamine pH 7.7 was applied to a Q-Sepharose column (10ml) equilibrated in the same buffer as described in Section 2.9. The fractions collected were assayed (reducing groups) for PG activity and

A



B



C

FIG. 6.6: FRACTIONATION OF POLYGALACTURONASE ISOENZYMES BY ANION EXCHANGE CHROMATOGRAPHY

A. A sample of ripe separated zone (151 daa, 0.9g fresh wt) was applied to the anion exchange column (Q-Sepharose) and washed in 20mM triethanolamine buffer pH 7.7. The PG enzyme was eluted by lowering the pH with histidine buffer pH 5.0. Fractions were assayed; 3 peaks of PG activity were obtained. The increased absorbance in fraction 45 represented the pectin/reducing sugar in the extract which eluted with 500mM NaCl/200mM histidine.

B. The fractions with PG activity (Peaks 1 and 2) were pooled and concentrated in an ultrafilter (3kD MWCO). They were then fractionated by IEF and the gels either silver-stained for protein or stained for activity with TTC.

C. After anion exchange chromatography, the pI 6.2-6.4 isoenzymes often had a "halo" of other bands of PG activity, possibly this was due to small changes in the enzyme structure that occurred during experimental procedures.

a major peak of activity was eluted from the column by lowering the pH with 50mM histidine pH 5.0 (Fig. 6.6A, Peak 1). A further peak of activity eluted with 100mM histidine pH 5.0 (Peak 2); a very small peak with 200mM histidine (Peak 3); and the pectins in the sample finally eluting with 500mM NaCl.

Fractions from the main peak of activity (Peak 1) were pooled, concentrated by ultrafiltration and applied to isoelectric focusing gels (Section 2.7). After electrophoresis and staining for reducing groups with TTC (Section 2.7.1) Peak 1 was shown to consist of 2 bands of activity (Fig. 6.6B), and when compared with IEF standards (see Figs. 6.7 and 7.3) these were located at about pI 6.2-6.4. The smaller peaks of activity from this column (Peaks 2 and 3) were also pooled and concentrated. Again, electrophoresis on IEF gels and staining for activity and protein showed that all these were isoenzymes with the same pI 6.2-6.4 (Fig. 6.6B). Sometimes, other "bands" of activity were seen to halo the two major bands of PG activity, and this was thought to represent small changes to the enzyme structure that may have occurred during the experimental procedures causing focusing slightly above and below the major bands at pI 6.2-6.4 (Fig. 6.6C).

The pI 6.2-6.4 PG isoenzymes were present in extracts of separated zone but were they present in extracts of the separated tepal base? This was examined in an extract of tepal base 1 from ripe fruit which had separated 1+4/5. The extract was prepared from a large amount of tissue (4g) and then applied to the anion exchange column. It was eluted by the same procedure used for extracts of zone. The fractions were incubated for a long period (20h) in order to detect a low activity. However, no activity at all was detected in the fractions which should have contained the pI 6.2-6.4 isoenzymes (there was an increase in absorbance in the final 500mM NaCl elution but this was due to pectin/reducing sugars in the extract). This confirmed the absence of the pI 6.2-6.4 isoenzymes in tepal base 1 extracts, since they were not visualised by staining either for protein (silver) or activity (TTC) on IEF gels (see Fig. 6.5A & B).

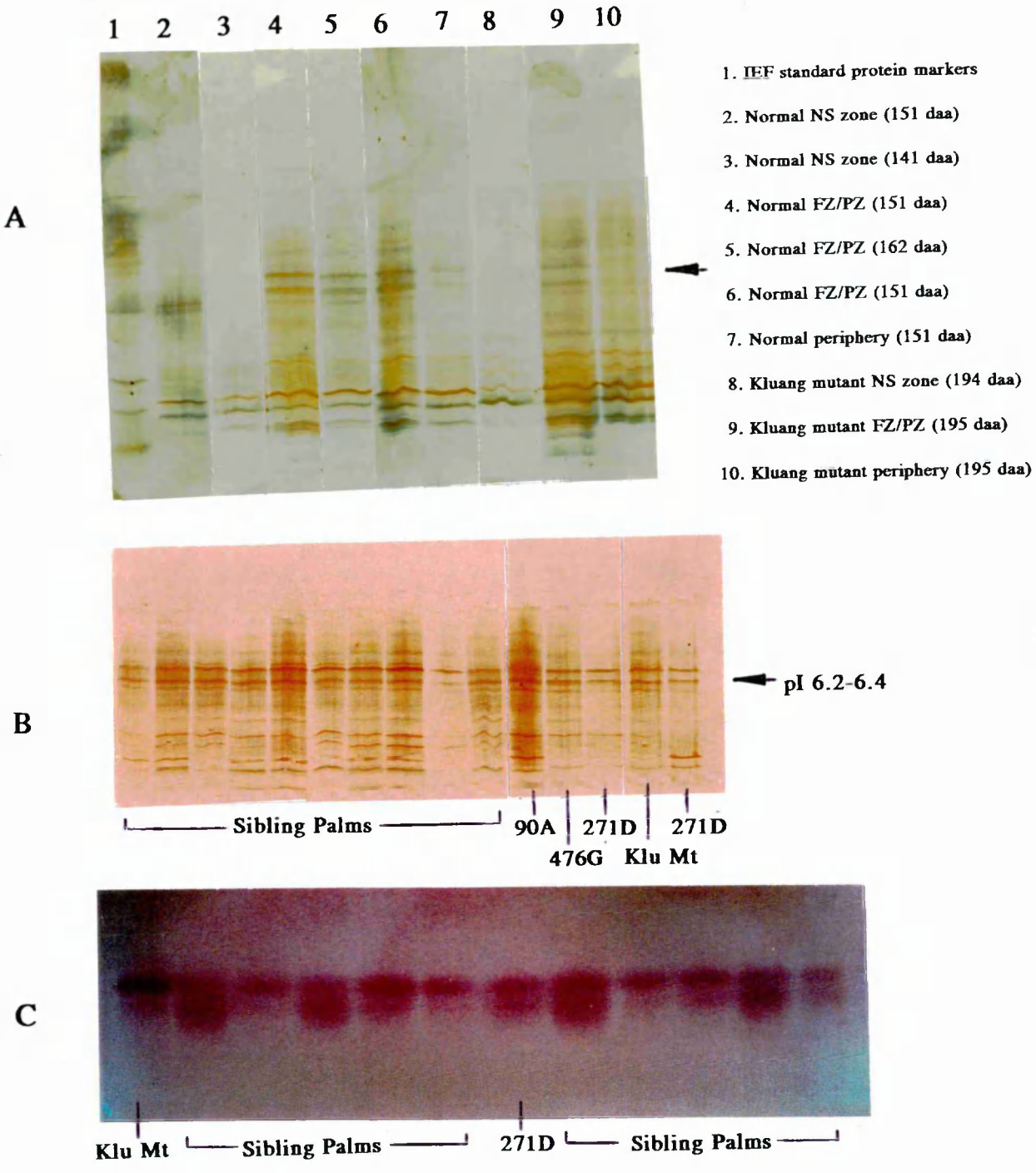


FIG. 6.7: THE PI 6.2-6.4 POLYGALACTURONASE ISOENZYMES (after concentration of the pooled fractions from Peak 1 of the Anion Exchange Chromatography)

A. A comparison of normal and Kluang mutant unseparated zone, separated zone and periphery tissues. Samples were prepared from the same fresh weight and concentrated to the same volume; 30μl of concentrate was fractionated by isoelectric focusing (3.5-9.5) and gels were silver-stained for protein. The arrow indicates the position of the pI 6.2-6.4 isoenzymes.

B. Separated zone of the Kluang mutant, 10 siblings of the Kluang mutant, and 3 normal clones (90A, 476G & 271D). Samples were prepared as for (A) and after isoelectric focusing were silver-stained for protein. [The palm identification numbers of the siblings of the Kluang mutant are (from L to R) 40, 36, 2, 7, 9, 19, 21, 22, 23 25.]

C. Extracts of (B) fractionated by isoelectric focusing and stained for activity (TTC) (90A and 476G are omitted). [The palm identification numbers of the siblings of the Kluang mutant are (from L to R) 25, 23, 22, 21, 19, 9, 7, 2, 36, 40.]

Samples of enzyme extracts of both normal and the Kluang mutant separated zone, unseparated zone and fruit periphery, were fractionated by the same method. The fractions with PG activity in the 50mM NaCl elution were pooled and concentrated by ultrafiltration and fractionated by isoelectric focusing in the same experiment. The gel was then cut and silver-stained for protein. Figure 6.7A (arrow indicates position of the pI 6.2-6.4 isoenzymes) shows that the Kluang mutant separated zone extract (Lane 9), after anion exchange chromatography, now had both bands of PG present (compare with normal separated zone, Lanes 4, 5 and 6). In addition, ten sibling palms of clone DxP5 (the same clone as the Kluang mutant), clones 271D, 90A, 476G and the Kluang mutant were analysed; it appeared that there was some variation in the relative amounts of these two PG isoenzymes extracted (Fig. 6.7B and C). The pI 6.4 isoenzyme generally had the highest activity.

The Kluang mutant zone extract had been partially cleaned of the larger pectins by filtration through a 100K ultrafilter and this filtrate further concentrated in a 3K ultrafilter. The small volume of enzyme extract remaining in the 100K ultrafilter containing the higher molecular weight pectins was then applied to an anion exchange column (Q-Sepharose) to remove the pectins. Elution of PG enzyme (by lowering the pH to 5.0 with 50mM histidine buffer) should then reveal if the pI 6.2 enzyme was initially present in the Kluang mutant and had been preferentially adsorbed to the water-soluble pectin in the

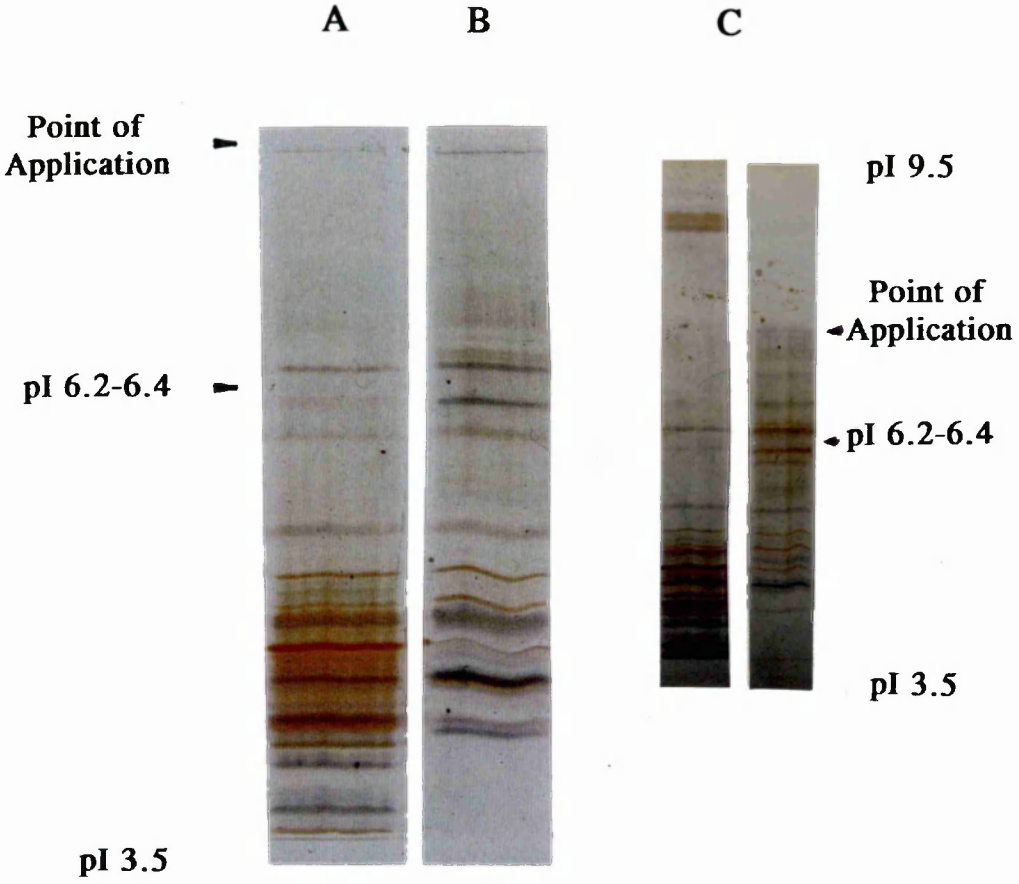


FIG. 6.8: THE PI 6.2-6.4 POLYGALACTURONASE ISOENZYMES IN THE KLUANG MUTANT SEPARATED ZONE

A. The pectins in the enzyme extract of separated zone tissue (167 daa) were removed by ultrafiltration (100kD MWCO) and the filtrate containing the PG enzymes was concentrated in a 3kD ultrafilter. After fractionation on IEF gels, only the pI 6.2 PG isoenzyme was visualised when the gel was silver-stained for protein.

B. The pectins remaining in the 100kD ultrafilter were applied to the anion exchange column (Q-Sepharose), washed with buffer and eluted as described in Section 2.9. The 50mM histidine pH 5.0 eluted fractions with PG activity were pooled, concentrated and fractionated by IEF (pI 3.5-9.5). Two bands with PG activity were now present at pI 6.2-6.4.

C. Another sample of the Kluang mutant separated zone (150 daa) was analysed as in (A) and (B) above, and the same result was obtained.

extract. Figure 6.8 shows that both the pI 6.4 and pI 6.2 PG isoenzymes were indeed present in the Kluang mutant and both had activity (see also Fig. 6.7C). This indicates that although the original zone tissue was extracted in high salt (1M NaCl), the pI 6.2 PG isoenzyme remained tightly bound to the pectin present in the mutant separated zone. Since this sequence of abscission changes occurs only after harvesting, and not in the non-abscinding fruit whilst on the palm, the post-harvest separation of fruit analysed here may involve the initiation of new gene expressions including polygalacturonase which do not occur on the palm and which permit separation of the mutant by a pathway different from that in normal fruit. However, whilst the pectins and/or cell walls in the separation zone of the Kluang mutant may be different from those in separation zones of normal fruit, the pI 6.2-6.4 PG isoenzymes of the zone are not.

Ali & Brady (1982) noted a more successful purification of endo-PG in the tomato with cation exchange columns: on anion exchangers the prolonged exposure to a pH above 7 resulted in a substantial loss in endo-PG activity. When an enzyme extract of separated zone was assayed in 20mM triethanolamine pH 7.7 or 50mM histidine pH 5.0, activity was only 22% and 28% respectively of that in 100mM acetate pH 5.3. The high activity of pI 6.2-6.4 isoenzymes (on activity gels) from separated zone showed that they appeared to be unaffected by the triethanolamine and histidine buffers and, if oil palm PG was similar to tomato PG, this suggests that these isoforms may **not** be endo-acting. In order to determine whether PG isoenzymes other than the pI 6.2-6.4 were present in separated zone extracts of oil palm, further fractionations were carried out by cation exchange chromatography.

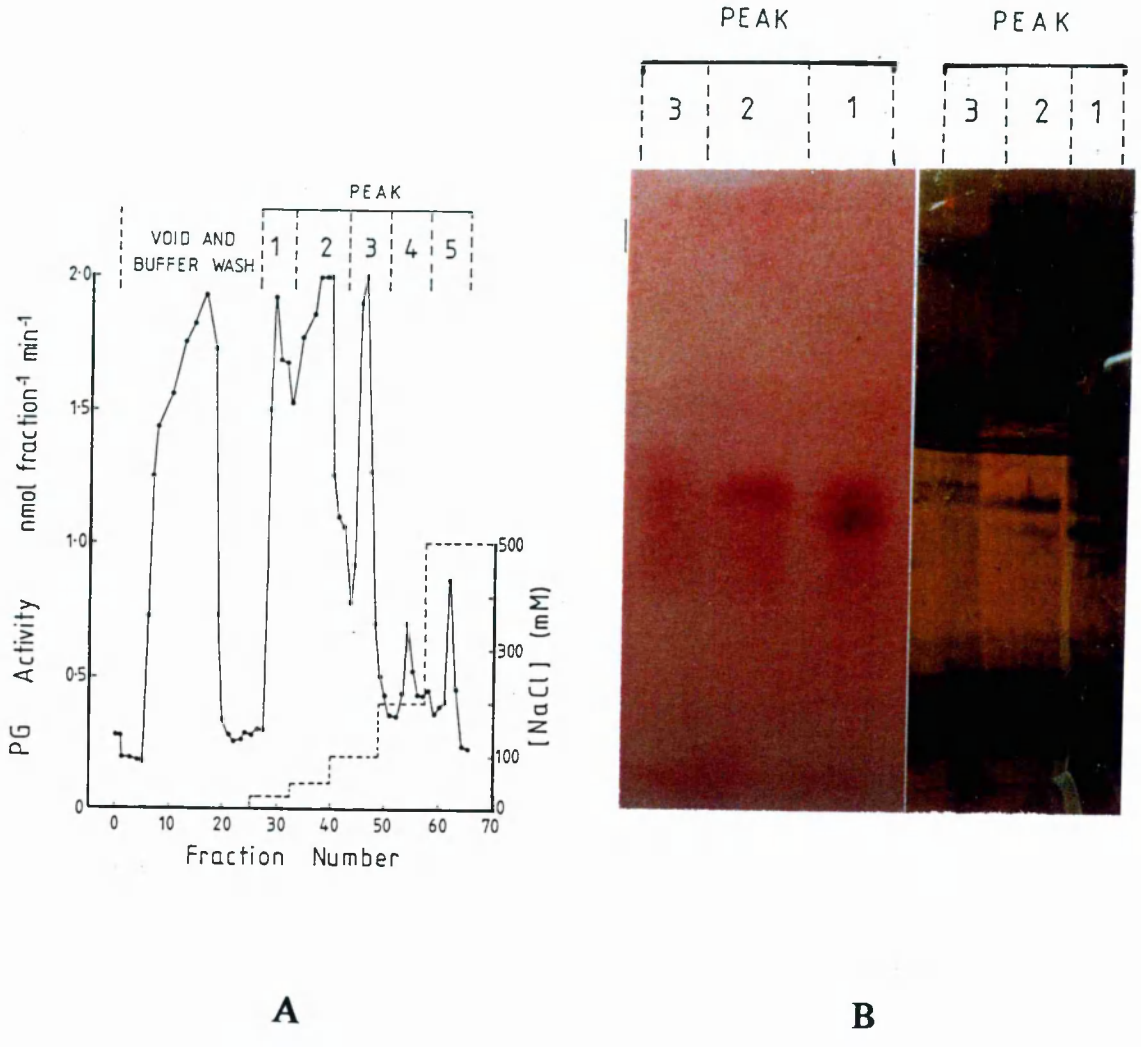


FIG. 6.9: FRACTIONATION OF POLYGALACTURONASE ISOENZYMES BY CATION EXCHANGE CHROMATOGRAPHY

A. A sample of separated zone (155 daa; 0.8g fresh wt) was applied to a cation exchange column (Express-Ion C), washed with buffer and eluted with a step-wise increase in NaCl concentration. Fractions were incubated with substrate (18h) and 3 main peaks of activity were obtained; 25mM (Peak 1), 50mM (Peak 2), and 100mM (Peak 3) NaCl elutions. The fractions from each peak were pooled, concentrated and desalted in a 3kD ultrafilter, each to the same volume.

B. Peaks 1, 2 and 3 were fractionated by IEF (pI 3.5-9.5). The gels were stained for activity (TTC) and protein (silver-stained). The concentrated sample of each peak (20μl) also was assayed for PG activity (Peak 1 contained 126.5nmol/h^{-1}; Peak 2: 103.9nmol/h^{-1}; and Peak 3: 84.9nmol/h^{-1}).

6.3.2 Isoenzymes Fractionated by Cation Exchange Chromatography

The samples in 20mM acetate buffer pH 5.5 were applied to a column (17ml) of Express Ion C (Whatman) equilibrated in the same buffer. Elution was with a step-wise increase in sodium chloride concentration; 25mM, 50mM, 100mM, 200mM and 500mM NaCl prepared in 20mM acetate pH 5.5. Fractions were collected and assayed for PG activity (reducing groups). The anionic pectins in the sample did not bind to the column and eluted in the void volume and first wash with buffer only. Initially, for a separated zone extract, 3 major peaks of activity were obtained (Fig. 6.9A) with the 25mM, 50mM and 100mM NaCl elution. Figure 6.9B shows that (after the fractions of these peaks were pooled, concentrated by ultrafiltration, fractionated by IEF electrophoresis and stained for activity with TTC) the 25mM NaCl eluted fraction contained both pI 6.2 and 6.4 isoenzymes; the 50mM NaCl eluted fraction was enriched with the pI 6.4 isoenzyme; and in the 100mM fraction, whilst there was some activity in the pI 6.0-6.5 region, this low activity on the gel did not correspond to the high activity measured by enzyme assay in the concentrated fractions of the 100mM peak (84.9 nmol h^{-1} compared with 126.5 nmol h^{-1} and 103.9 nmol h^{-1} in the 25mM and 50mM NaCl eluted fractions respectively). Also, small peaks of activity were present in the 200mM and 500mM NaCl eluted fractions.

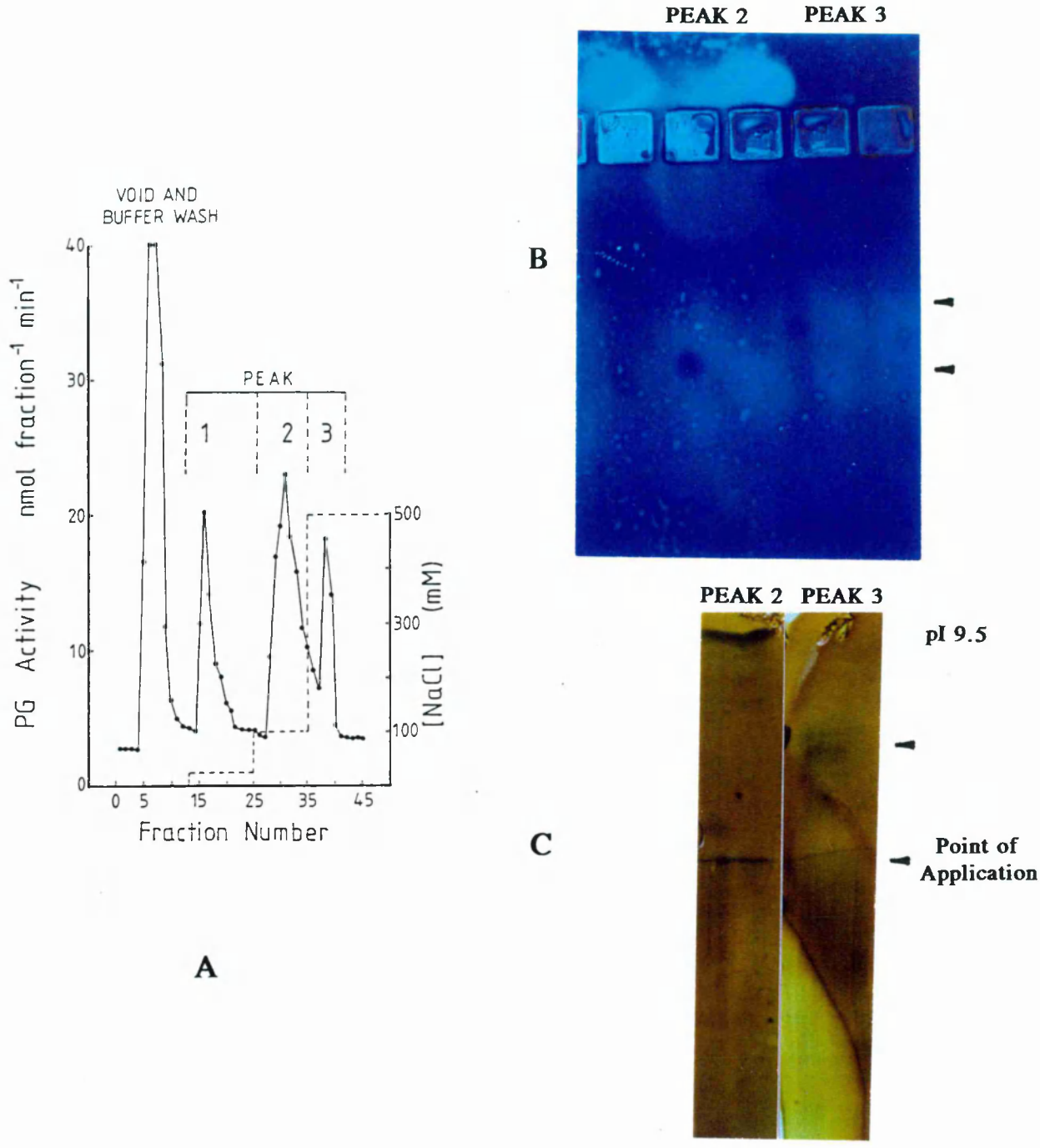


FIG. 6.10: CATION EXCHANGE CHROMATOGRAPHY AND ELECTROPHORESIS OF POLYGALACTURONASE ISOENZYMES

A. An extract of ripe separated zone (4ml containing 500mg fresh wt; 152 daa) was applied to a cation exchange column (Express-Ion C), washed and eluted with step-wise increases in NaCl concentration. Fractions were assayed for PG activity (1h) and 3 peaks of activity were obtained.

B. The eluted fractions in Peak 1 (50mM NaCl); Peak 2 (100mM NaCl) and Peak 3 (500mM NaCl) were pooled, concentrated and desalted in a 3kD ultrafilter. Peaks 2 and 3 were then fractionated by native anionic electrophoresis (Cleangel, pH 8.9). After electrophoresis, the gel was equilibrated to pH 5.5, incubated with an overlay of PGA substrate (18h) and stained with methylene blue.

C. The concentrate of Peaks 2 and 3 as in (B) above (30μl) were fractionated by isoelectric focusing (pI 3.5-9.5) and silver-stained for protein.

Based on these results, the elution conditions were then modified: 50mM, 100mM and 500mM NaCl fractions were eluted and collected. The 50mM NaCl eluted fraction contained most of the pI 6.2 and 6.4 isoenzymes (as shown in Fig. 6.9). The 100mM and 500mM NaCl eluted fractions (Fig 6.10A, Peaks 2 and 3) were collected, concentrated and desalted by ultrafiltration. To visualise the PG isoenzymes in these two concentrates, anodic native gel electrophoresis (Cleangel, Pharmacia) with methylene blue staining for activity was used, rather than fractionation on IEF gels. Although the pH of the native gel was 8.9, Fig. 6.10B shows that the concentrate of the 100mM NaCl eluted fraction (Peak 2) did not lose all activity at this pH. The PG protein migrated toward the cathode and not into the gel itself indicating this isoenzyme had a basic pI greater than that of the gel (pH 8.9). There was also a smear of activity from protein which had migrated into the gel (which may represent some pI 6.2-6.4 enzyme in this fraction). In the 500mM NaCl eluted fraction (Peak 3), there was some activity from protein which migrated into the gel. These two concentrates (100mM and 500mM NaCl) were then fractionated on IEF gels and, after silver-staining (Fig. 6.10C), showed a protein band at pI 9.5 in the 100mM fraction and two faint protein bands in the 500mM fraction. However, even staining with silver was not sufficiently sensitive to detect the low amount of protein in the 500mM NaCl eluted fraction (Peak 3) from an individual extraction and fractionation. Therefore, twelve different samples of ripe separated zone tissue (each of 500mg) were extracted and fractionated by cation exchange chromatography. The fractions containing activity in Peak 2 and Peak 3 were pooled, concentrated and desalted (3kD ultrafilter).

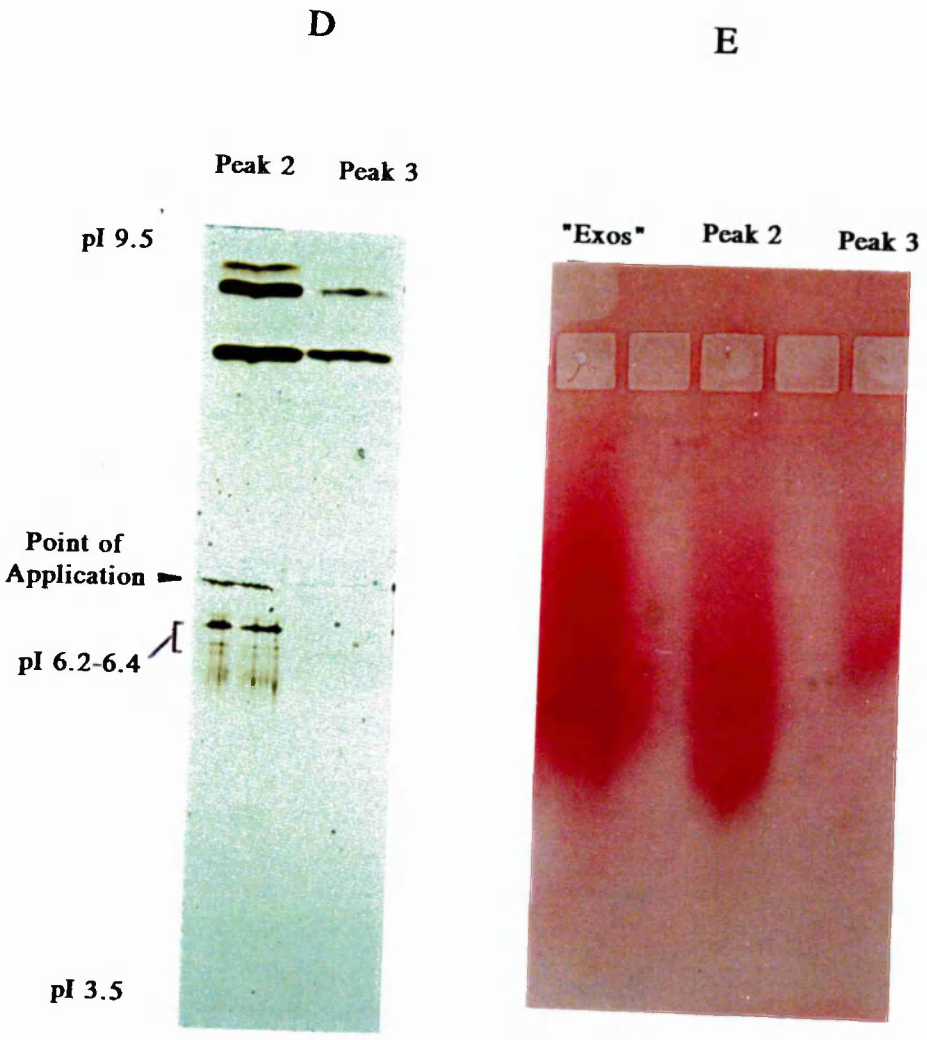


FIG. 6.10: CATION EXCHANGE CHROMATOGRAPHY AND ELECTROPHORESIS OF POLYGALACTURONASE ISOENZYMES

D. The concentrates of the 100mM (Peak 2) and the 500mM (Peak 3) NaCl elutions from 12 different separated zone samples were fractionated by isoelectric focusing (pI 3.5-9.5). The gels were silver-stained for protein.

E. A concentrate of fractions containing PG activity eluted from the **anion** exchange chromatography (pI 6.2-6.4; exo-activity) was compared with the 100mM (Peak 2) and 500mM (Peak 3) NaCl elutions from the **cation** exchange chromatography (shown in D above).

These concentrates were then fractionated on IEF gels (pI 3.5-9.5). Figure 6.10D shows silver-stained IEF gels of the 100mM (Peak 2) and 500mM (Peak 3) NaCl eluted fractions; 3 major protein bands at a basic pI (~9.0-9.4) were present in the 100mM eluted fraction and 2 bands in the 500mM eluted fraction. Cathodic native gel electrophoresis (Cleangel, Pharmacia) at pH 5.5 was used to determine which of these bands contained PG activity. A concentrated extract (from the anion exchange column) containing the pI 6.2-6.4 (exo-enzymes) was compared with the 100mM and 500mM NaCl eluted concentrates from the cation exchange chromatography. Exo-activity produces reducing sugar monomers which react strongly with TTC to give an intense pink stain. Figure 6.10E confirms that Peak 2 (100mM NaCl elution) and Peak 3 (500mM NaCl elution) from the cation exchange chromatography contained basic isoforms with PG activity. Since discontinuous native polyacrylamide gel electrophoresis fractionates proteins by a combination of size and charge (Hedrick & Smith, 1968), in these gels the pI 6.2-6.4 isoenzymes appear to fractionate between the two basic isoforms (Fig. 6.10E). However, the protein bands are very close together and it is difficult to clearly resolve the different PG isoforms when the gel is stained for activity (due to the diffusion of product).

The oil palm abscission zone basic PG isoenzymes, that is, those found in both the 100mM NaCl eluted fractions (pI ~9.3) and the 500mM NaCl eluted fractions (pI ~9.0) appear to have similar pIs as the tomato fruit basic endo-enzymes: PG2 (pI 9.4) and PG1 (pI 8.6) (Ali & Brady, 1982). For comparison, an extract of tomato pericarp (Gardeners Delight, home grown) was prepared by the same methods as those used for oil palm extracts. The extract was fractionated by cation exchange chromatography; no activity was obtained in the 50mM NaCl eluted fractions even after long incubations (18h) but very high activity was present in the 100mM and 500mM NaCl eluted fractions. All fractions with activity were pooled, concentrated and desalted (3kD ultrafilter). Fractionation on

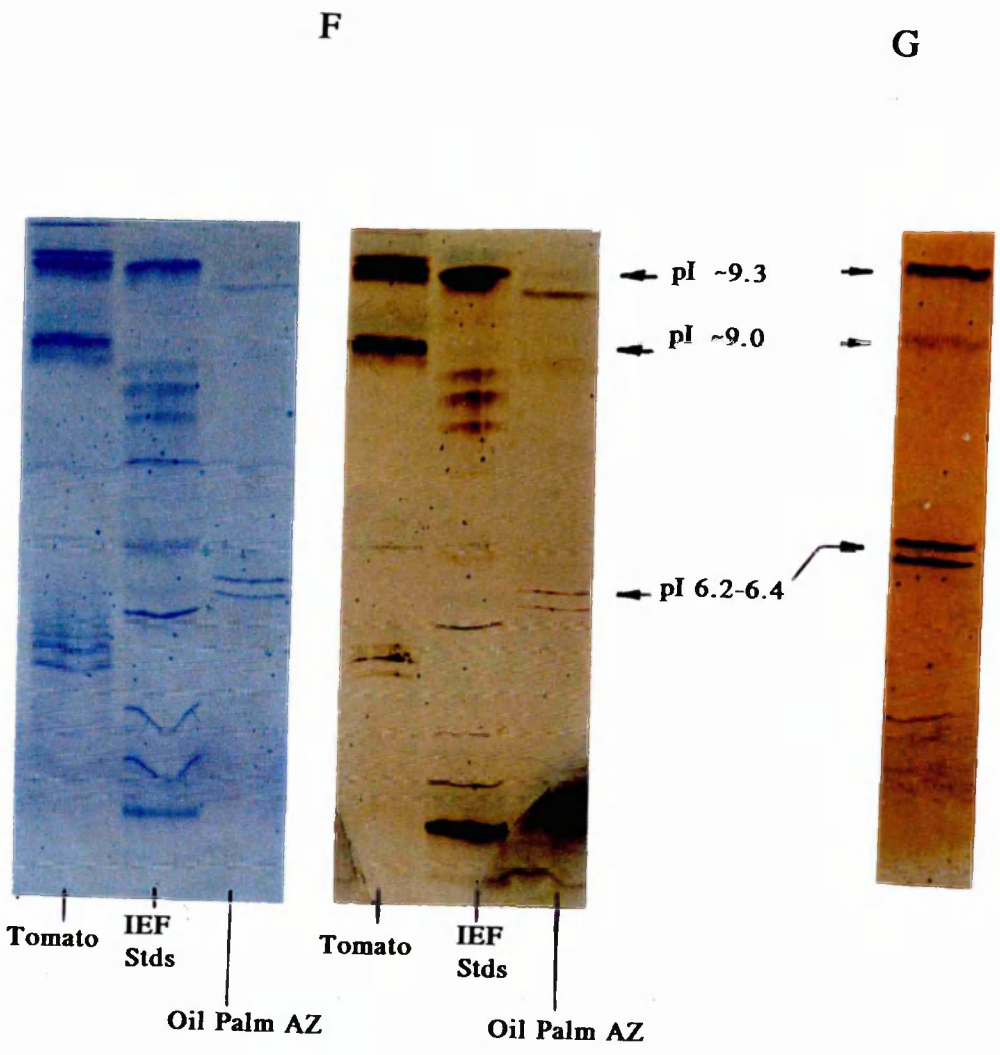


FIG. 6.10: CATION EXCHANGE CHROMATOGRAPHY AND ELECTROPHORESIS OF POLYGALACTURONASE ISOENZYMES

F. The pIs of all the PG isoenzymes of ripe separated zone (in the 500mM NaCl eluted concentrate of (G below) were compared with tomato (extracted and fractionated by cation exchange chromatography in the same way as for the oil palm extracts) and an IEF standard protein mix (Sigma). Gels were stained for protein with Coomassie blue and silver.

G. Ripe separated zone tissue (147 daa) was extracted at pH 1.6 (with 1M NaCl), desalted (Biogel) and fractionated by cation exchange chromatography. The fractions containing PG activity (500mM NaCl elution) were pooled, desalted and concentrated (3kD ultrafilter); 30μl of concentrate was fractionated by isoelectric focusing (pI 3.5-9.5). The gel was silver-stained for protein.

IEF gels and silver-staining showed two basic protein bands at pI ~9.0 and pI ~9.4 (Fig. 6.10F). These no doubt contained the PG1 and PG2 protein. The concentrates of separated oil palm abscission zone and ripe tomato pericarp were compared with an IEF standard protein mix (Sigma). Figure 6.10F also shows that the oil palm basic PG isoforms fractionate at similar pIs to those of the tomato.

The IEF standard protein markers contain three isoenzymes of lentil lectin with pIs, calculated by Sigma, of 8.2, 8.6 and 8.8. Comparison with the lentil lectin isoenzymes gives values for the tomato PG isoforms of pI ~9.0 and 9.4. Since the literature values for tomato PG are pI 8.6 and 9.4 (Ali & Brady, 1982), it may be that the conditions used in these experiments causes the lentil lectin proteins to fractionate at slightly lower pIs than the values given by Sigma. It is interesting to note that both Bio-Rad and Pharmacia quote lower pI values for the lentil lectin isoenzymes in their IEF standard protein markers for pI 3.6-9.3 (Bio-Rad gives values of 7.8, 8.0, 8.2; Phamacia gives values of 8.15, 8.45, 8.65). The pIs quoted by Pharmacia and Bio-Rad for all the other proteins in their IEF standard protein markers are very similar to each other (and to those quoted by Sigma). The calibration curve (Fig. 2.8, page 65A) of the Sigma standard IEF protein markers indicates that the lentil lectin proteins may fractionate at lower pIs than those given by Sigma.

When tomato fruit has been extracted in water at pH 1.6, PG2 is the major isoenzyme obtained (Pressey, 1988). Again, for comparison, ripe separated abscission zone tissue was extracted at pH 5.0 and pH 1.6 (with 1M NaCl). The extracts were desalted by gel filtration with a simultaneous pH change (50mM acetate, pH 5.5) and then fractionated by cation exchange chromatography. The elution profile of the pH 5.0 extract was the same as that shown in Fig. 6.10A. However, for the pH 1.6 extract, activity was obtained only in the 500mM NaCl eluted fractions. These fractions were pooled, concentrated/desalted (3kD ultrafilter), and fractionated on IEF gels. The gels were silver-stained for protein and Fig. 6.10G shows that, in addition to the high pI enzymes, the pI 6.2-6.4 isoenzymes are also present. The low pH extraction has caused the pI 6.2-6.4 isoenzymes to bind more tightly to the cation exchange support, eluting with 500mM NaCl (instead of 50mM NaCl). The two basic isoforms, visualised at pI ~9.3 and pI ~9.0, have changed in their relative concentration; there is more pI ~9.3 protein and considerably less pI ~9.0 protein (compare Fig. 6.10B and Fig. 6.10G). Thus, the oil palm abscission

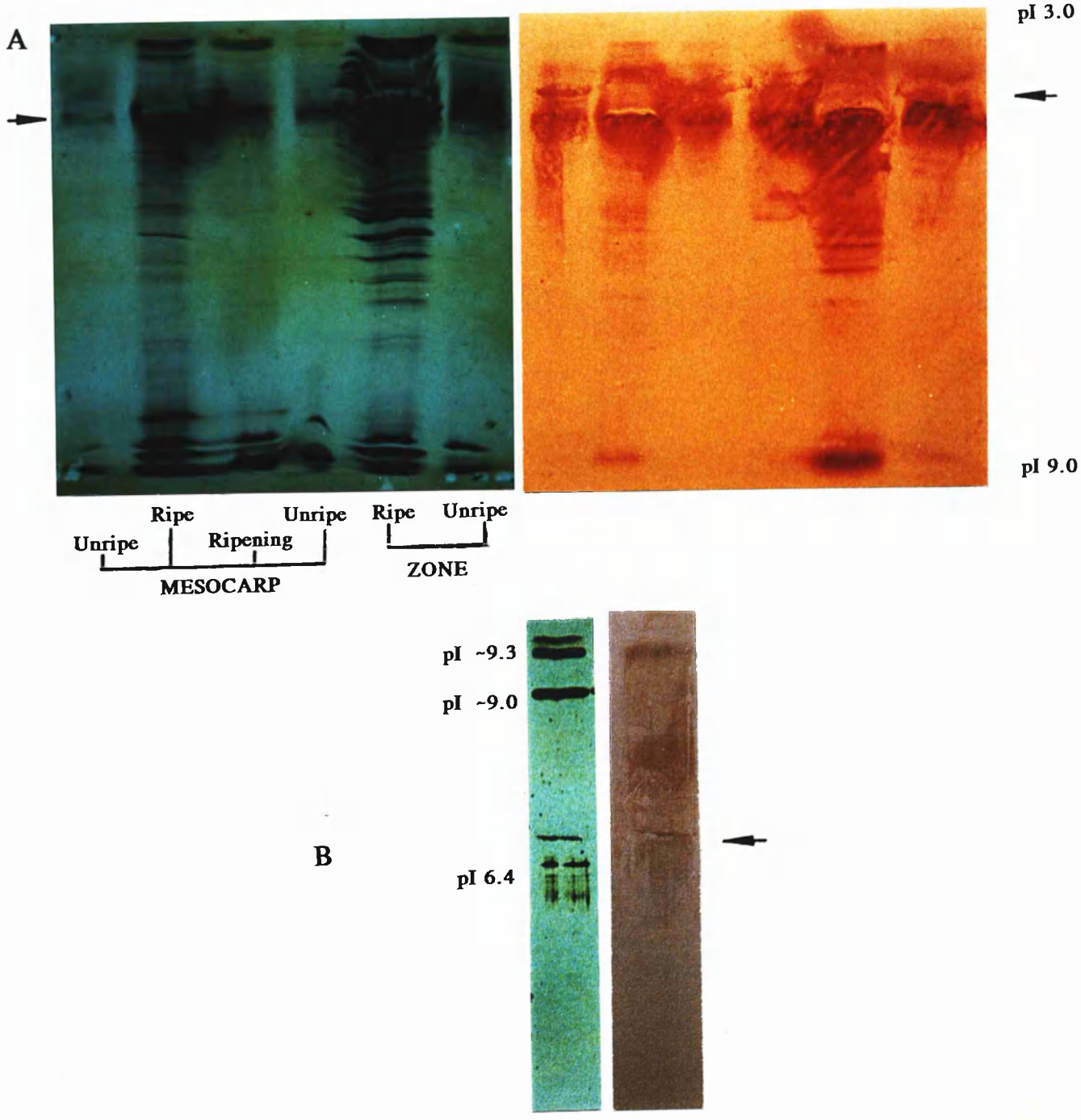


FIG. 6.11: IMMUNORECOGNITION OF OIL PALM POLYGALACTURONASE WITH THE TOMATO FRUIT ENDO-POLYGALACTURONASE ANTISERUM

A. Enzyme extracts (4μl, clone 90A) were fractionated on two Phastgels by isoelectric focusing (pI 3.0-9.0). One gel was silver-stained for protein; the other gel was used in a Western Blot and probed with antiserum to tomato fruit endo-PG.
Unripe = 106 daa; Ripening = 126 daa; Ripe = 158 daa.
Arrow indicates point of application.

B. The concentrate from the 100mM NaCl elution of the cation exchange chromatography was probed with antiserum to tomato fruit endo-polgalacturonase (a different sample of antiserum to that used in (A) above).

zone basic PG isoenzymes also appear to behave in a similar way to the tomato fruit PG when extracted at low pH.

A polyclonal antibody to tomato fruit endo-PG kindly donated by Dr G. Tucker was used to probe for recognition of the oil palm abscission zone PG (as described in Section 2.8). Figure 6.11A shows a Western blot of a Phast gel and in the separated abscission zone the tomato antibody did show recognition of the pI 6.2-6.4 isoenzymes, the pI 9.3 and perhaps the pI ~9.0 endo-enzymes. The blot gave no recognition of the pI 6.2-6.4 isoenzymes in the mesocarp and the unseparated zone but some recognition of the basic pI isoform was observed for each of these tissues. This antibody appears to recognise proteins other than those with PG activity, so another tomato fruit endo-PG antibody (also provided by Dr G. Tucker) was used on concentrated desalted fractions of the 100mM and 500mM NaCl elutions from the cation exchange column. Recognition of the pI~9.3 isoenzyme was obtained in both the 100mM and 500mM eluted fractions (Fig. 6.11B shows the 100mM fraction).

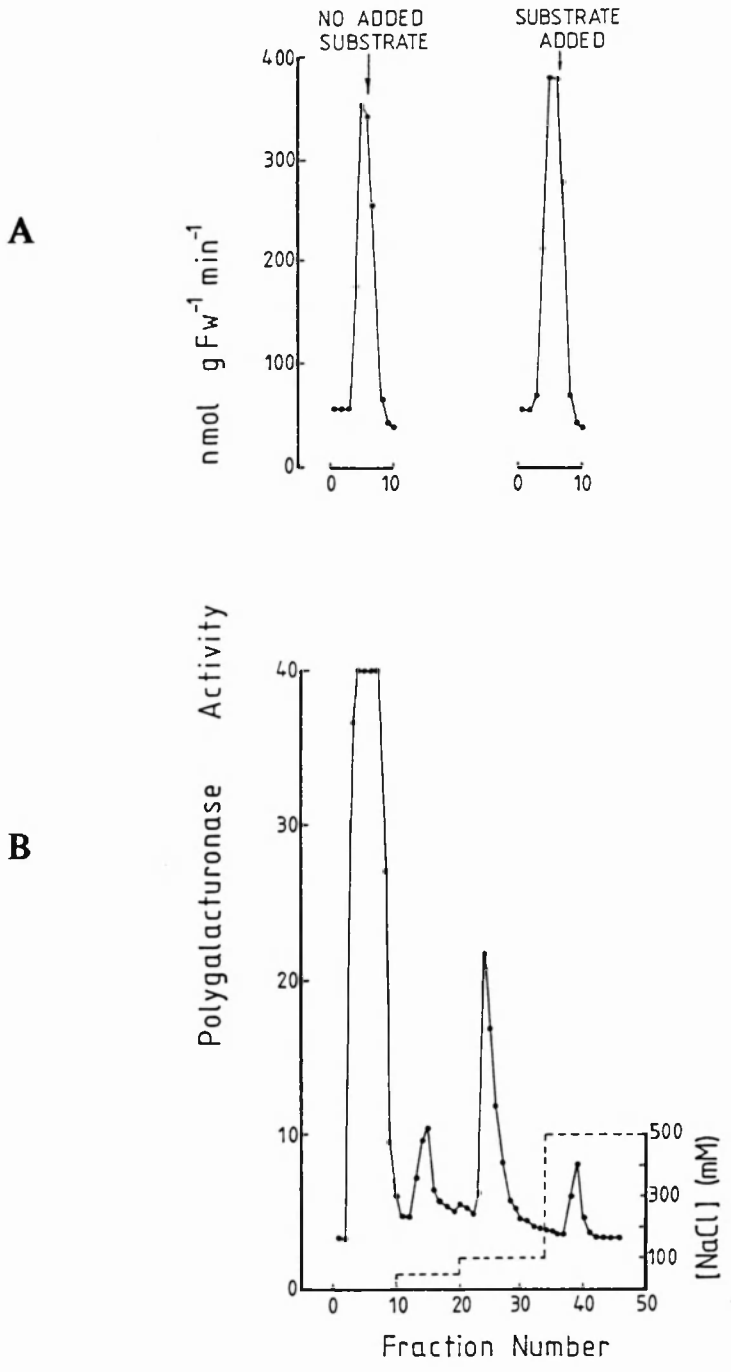


FIG. 6.12: THE VOID FRACTIONS (1-10) FROM CATION EXCHANGE CHROMATOGRAPHY

The void fractions of enzyme extracts (separated zone, unseparated zone, mesocarp and tepal base) had a very high absorbance when assayed for reducing groups (cyanoacetamide method of Gross, 1982).

A. The void fractions of a separated zone extract (10μl of each fraction) were incubated without and with PGA substrate. Additional PGA substrate does not markedly increase the absorbance. Therefore, the absorbance is due mainly to pectin/reducing sugars and endogenous enzyme activity.

B. The void fractions of (A) above were pooled and re-chromatographed on the cation exchange column. Some additional PG enzyme was desorbed from the pectin. This showed that not all the PG enzyme is removed from the pectin in the extract by cation exchange chromatography and the association of PG enzyme with the pectin is strong.

The fractions of the void volume of the cation exchange column had a very high absorbance in the absence of added substrate. This was thought to be mainly pectin; the negatively charged uronic acids do not adsorb to the cation exchange matrix. Figure 6.12A shows that fractions 4-8 of the control, incubated with **no** substrate, contributes almost the same the nmol reducing groups measured in the fractions with substrate. Hydrolysis of this endogenous substrate may occur by enzymes other than PG which are not adsorbed to the column at pH 5.5, although some of the PG isoenzymes are present. This was confirmed by re-chromatography of the void fractions on the cation exchange column. Again, activity was obtained in the 50mM, 100mM and 500mM NaCl eluted fractions (Fig. 6.12B) in similar proportions but at lower levels to the first fractionation. Thus, it is not possible to completely remove all of the PG isoenzymes from the pectin with chromatography on a cation exchange matrix, and this suggests tight binding of the PG isoenzymes to the pectin in the sample.

Whilst the cation exchange fractionation appeared to give good separation of the isoenzymes, the 50mM, 100mM and 500mM NaCl fractions from step-wise elution did not represent "pure" proteins. Some PG enzyme from the preceding fraction would no doubt be present. Thus, the 100mM NaCl eluted fraction can be enriched in the pI ~9.3 isoenzyme and the 500mM NaCl eluted fraction enriched in the pI ~9.0 isoenzyme.

Since the slow post-harvest separation of the Kluang mutant is not due to any differences in the pI 6.2-6.4 PG isoforms, the more basic pI isoforms were examined. Enzyme extracts of unseparated and separated zone, mesocarp and tepal base 1 tissue from the normal and Kluang mutant were compared by fractionation with cation exchange

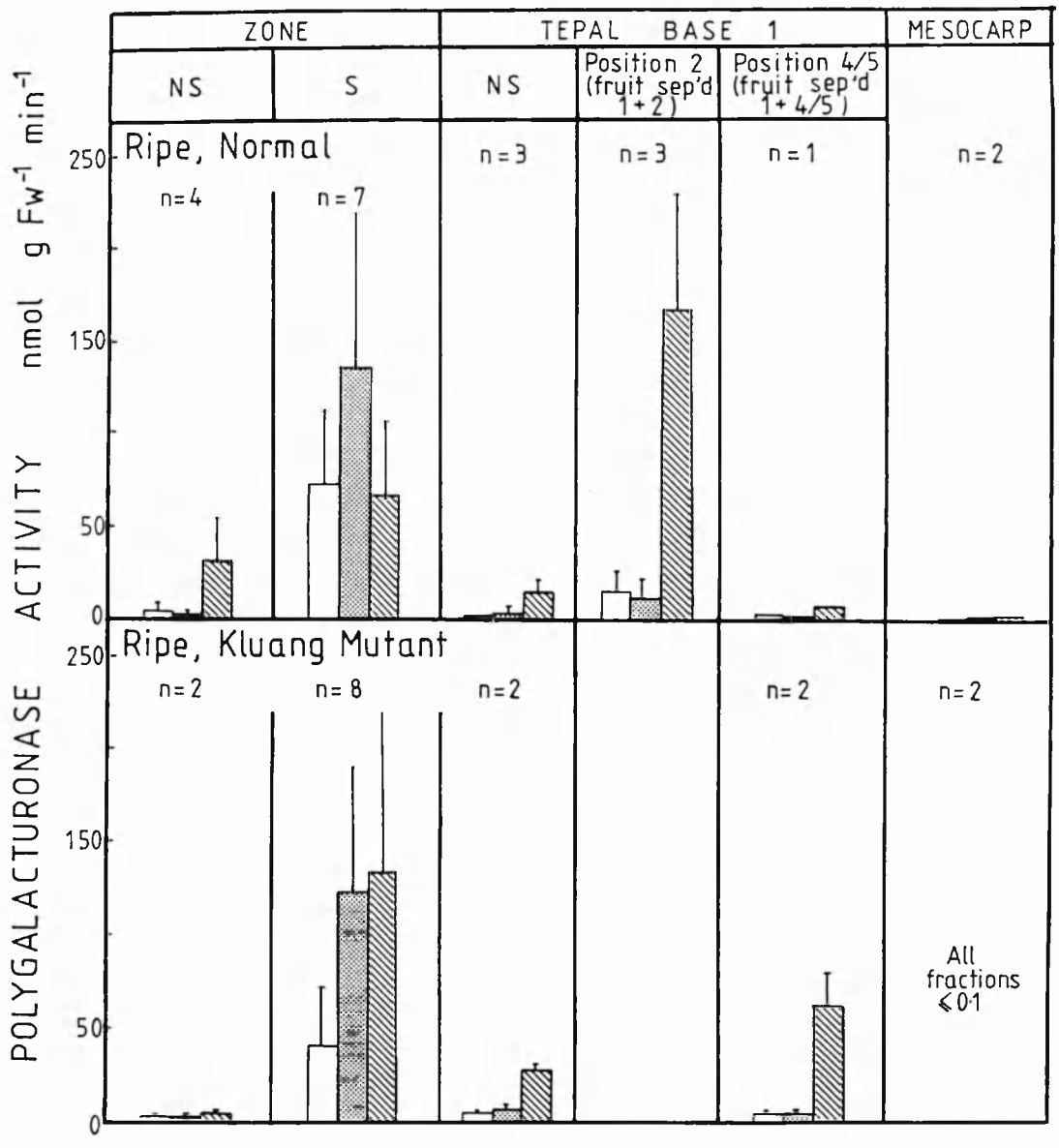


FIG. 6.13: THE POLYGALACTURONASE ACTIVITY IN FRACTIONS AFTER CATION EXCHANGE CHROMATOGRAPHY OF THE DIFFERENT TISSUES IN NORMAL AND KLUANG MUTANT FRUIT

The PG isoenzymes in the different tissues of the normal and Kluang mutant fruit were compared. Enzyme extracts of unseparated zone, mesocarp and tepal base 1 were prepared from 1g of tissue and separated zone, 500mg.

The total PG activity of fractions in the 50mM (Peak 1), 100mM (Peak 2) and 500mM (Peak 3) NaCl elutions from the cation exchange column were summed, and a mean and standard deviation calculated for the number of samples analysed.

☐ Peak 1 ▒ Peak 2 ▨ Peak 3

chromatography. Figure 6.13 shows that most of the PG activity in unseparated zones comprised the basic PG isoenzyme(s) in the 500mM NaCl eluted fraction. The very low activity in the 50mM NaCl eluted fraction (pI 6.2-6.4 isoenzymes) confirms the results of the enzyme assays and the isoenzyme pattern observed on the silver-stained IEF gel (Fig. 6.7A) where these isoenzymes were barely visualised and possibly were not present. As abscission proceeds, the greatest increase in PG activity in the zone occurred in the 50mM and 100mM NaCl eluted fractions. In tepal base 1 and mesocarp, the 500mM NaCl eluted fraction contained most of the PG activity. The PG activity in the 500mM eluted fraction of tepal base 1 from 1+2 separated fruit was very high and quite different from that in 1+4/5 separated fruit. Possibly, this PG isoenzyme is important in cell separation at Position 2/3.

6.4 PECTIN METHYLESTERASE ACTIVITY IN TISSUES OF THE NORMAL AND KLUANG MUTANT FRUIT

Pectin methylesterase (PME) activity in the oil palm was assayed by the method of Hagerman & Austin (1986), as described in Section 2.6.4. Activity is expressed as nmol galacturonic acid equivalents g Fw^{-1} min^{-1}.

PME is thought to work co-operatively with PG; PME demethylates the pectin so that PG can hydrolyse the galacturonic acid backbone. Whilst no differences in PG activity were observed between the normal and Kluang mutant fruit, the ^{13}C CP-MAS NMR spectra of the normal and Kluang mutant separated zone tissue (Section 3.5.3, Fig. 3.15, page 95A) suggested that there was more methylated pectin in the mutant tissue. PME activity was compared in the different tissues of normal and mutant fruit. Table 6.22 shows that in all tissues of the Kluang mutant, PME activity was much lower than in the normal.

	PECTIN METHYLESTERASE ACTIVITY nmol g Fw^{-1} min^{-1}	
TISSUE	**NORMAL**	**KLUANG MUTANT**
ZONE		
Ripe separated FZ/PZ	**2956 ± 169** n = 4 (2742-3133)	**882 ± 277** n = 5 (480-1109)
Ripe not separated	**1119** n = 1	**397** n = 1
MESOCARP		
Unripe	**2237 ± 256** n = 2 (2056-2418)	**580** n = 1
Ripening	**7658** n = 1	-
Ripe	**6681 ± 3382** n = 3 (3183-9933)	**505 ± 135** n = 5 (376-725)
TEPAL BASE 1		
Not separated from pedicel	**567 ± 179** n = 2 (440-693)	-
Separated 1+4/5	**1442 ± 150** n = 3 (1330-1613)	**468** n = 1
PEDICEL FIBRES	**1472** n = 1	-

Table 6.22: PME Activity in Enzyme Extracts of Zone, Mesocarp, Tepal Base 1 and Pedicel Tissues in Normal and Kluang Mutant Fruit.

During ripening, the activity of PME was reported to increase ~1.4 times in the tomato (Hobson, 1963) and in banana (Brady, 1976), there was only a small increase. An approximately 1.5 to 4.5-fold increase in PME activity was found in ripe oil palm mesocarp, compared with the unripe tissue. However, analysis of more samples is required. Huber (1983) reported that PME activity was inhibited by some cations and polyphenols/polyols, and these are known to be present in oil palm fruit tissue (Henderson & Osborne, 1990).

Fractions obtained from NaCl elutions (without buffer) in anion and cation exchange chromatography were assayed for PME activity by the addition of substrate and

Universal indicator at pH 6.8. However, a visual pH colour change is not sufficiently sensitive to reveal differences between the normal and Kluang mutant tissues. Detection of PME isoenzymes in cherry fruits by activity staining on IEF gels has been reported (Alonso *et al,* 1995) and this technique may reveal specific differences between the normal and the Kluang mutant.

6.5 SUMMARY AND DISCUSSION

6.5.1 Pectic Enzyme Activity in Different Tissues of the Oil Palm Fruit

Ethylene production in the mesocarp was not detectable in the fruit, even when ripe, until abscission. The increased level of ethylene was coincident with the appearance of the pI 6.2-6.4 PG isoenzymes (exo-enzymes). These were found only in abscission tissue and contributed to the greatly increased PG activity of ~20-40 fold. However, this increased activity did **not** occur in the mesocarp of separated ethylene-producing fruit; only the endo-PG and not the pI 6.2-6.4 isoenzymes have been found in the mesocarp. The ethylene regulation of the genetic expression and induction of PG synthesis in the mesocarp and zone tissues is, therefore, very different. Ripe oil palm fruit abscind within 24h and perhaps it may be even faster on the palm in its tropical environment. The separated abscission zone has about 50-100 times more PG activity than in ripe mesocarp and four PG isoenzymes have been identified in tissue extracts fractionated by cation exchange chromatography and isoelectric focusing; pI 6.2-6.4, pI ~9.0 and pI ~9.3.

The induction of PG enzymes and their subsequent activity in the pectin-rich zone is strong evidence that PG plays a major role in oil palm fruit abscission. Before Position 1 separates, the pI 6.2-6.4 isoenzymes are present in very low amounts and are barely visualised (Fig. 6.7). After fruit separate naturally, the exo-PG enzymes pI 6.2-6.4 are also present at Position 2/3 (Periphery, Fig. 6.7), although very high activity was not dectected. When the tepal whorl separates (Position 4/5), PG activity is very low (similar to levels in the mesocarp and pedicel) and the pI 6.2 and 6.4 isoenzymes have not been found in this separation position. The vascular connections are attenuated in Position 4/5 as they are in Position 1 (zone) and Fig. 3.10A & C (page 88A) show that in this position there is an area which is not stained by phloroglucinol. This is where separation occurs between the pedicel fibre tissue and tepal base. Therefore, the regulation of separation at

Position 4/5 must be different: the pI 6.2-6.4 isoenzymes are not induced and separation is not dependent on high levels of PG activity, unlike natural abscission at Position 1+2/3.

The PME activity in extracts of the separated zone and mesocarp was the opposite of the PG activity: in all samples analysed, the activity in the mesocarp was always higher than that in the zone. The Ni^{2+}/Na_2S staining of the unseparated abscission zone (Fig. 3.12, page 90A) suggested that there was more polygalacturonate than methylesterified pectin but even so, there was considerable PME activity in separated zone tissue. Steele *et al* (1997) have used antibodies to methylesterified and non-esterified pectic epitopes and have shown that during tomato fruit ripening, de-esterification of pectins (and deposition of PG2 protein) appears to occur in block-like domains within the cell wall which implies a strict spatial regulation of enzymic activities. It would be interesting to use the JIM5 (recognises a relatively unesterified pectic epitope) and JIM7 (recognises a relatively methylesterified pectic epitope) antibodies on unseparated, translucent and separating oil palm abscission zones and compare these with those of the Kluang mutant. This might help to determine the part PME activity plays in oil palm fruit abscission. However, the level of PME activity extracted and assayed does not necessarily reflect its activity *in situ*. Goldberg *et al* (1992) have shown that PME in mung bean hypocotyls has a high ionic sensitivity especially toward cations. The ionic microenvironment of the cell wall could thus regulate PME activity. It is also possible that PME could be regulated, *in vivo*, by a protein analagous to the ß-subunit of polygalacturonase isoenzyme 1 (PG1). A glycoprotein inhibitor of PME was isolated from ripe kiwifruit (Balestrieri *et al*, 1990) and was found to inhibit PME from all fruits assayed (orange, tomato, apple, banana, potato). In addition, whilst methylester hydrolysis by PME may occur *in vitro*, Fry (1986) has suggested that *in vivo* PME may be involved in transesterification reactions, resulting in the formation of uronoyl-sugar ester cross-links.

6.5.2 Comparison of Pectic Enzyme Activity in the Normal and Kluang Mutant Fruit Tissues

PG activity was essentially the same in tissues of the normal and Kluang mutant. The ripe Kluang mutant samples received have been 20-70 days older than normal fruit consignments. Although the mutant mesocarp had probably been producing ethylene for some time before arrival in the laboratory, PG activity was still low, confirming that there must be a different regulation of PG in the mesocarp compared with the zone. After

harvesting, zone separation in the Kluang mutant becomes possible and PG activity in extracts of these separated mutant zones was similar to that found in the normal.

Whilst PG levels in all the tissues of the Kluang mutant were similar to those in normal tissues, the PME activity was markedly lower in both the mesocarp and the zone (Table 6.22). Experiments with transgenic antisense PME tomatoes have shown that low PME activity (< 10% of wild type) causes an almost complete loss of integrity in the fruit during senescence (Tieman & Handa, 1994). The lower PME activity in the Kluang mutant mesocarp - about 5-15% of normal levels (Table 6.22) - may contribute to the deterioration observed in this tissue (Figs. 3.1-3.3, pages 78A-79) and to what seems to be a loss of tissue integrity in the mesocarp of older fruit. In the separated zone tissue of the Kluang mutant, the PME activity was about 30% of normal levels. The ^{13}C CP-MAS NMR spectrum of separated zone tissue in the Kluang mutant (Fig. 3.15) showed that the resonance for the methylesterified carbon (at ~54 ppm) was more intense than that observed for normal separated zone tissue; that is, less de-esterification may have occurred. In addition, in extracts of the Kluang mutant separated zone, the pI 6.2 PG isoform was preferentially retained by the water soluble pectins in the extract (Fig. 6.8). Since anion exchange chromatography caused desorption of this isoenzyme from the pectin, the tight binding was ionic rather than covalent. Again this indicates that the water-soluble pectin in the mutant zone is different from that in the normal. The overall differences in pectin metabolism found in the Kluang mutant could possibly be initiated by the "disabled" cellulase enzyme (Section 5.2.2, page 124). Another explanation may be that the low PME activity in the Kluang mutant extracts is due to retention of the enzyme by cell wall material (as occurred with the pI 6.2 PG isoenzyme); the majority of the PME would then not be solubilised during extraction and would be sedimented in the pellet. Glover & Brady (1995) have described the tight association of PME with pectic polymers in mature unripe peach fruit which co-precipitated when tissue extracts were dialysed, resulting in an underestimate of PME activity, whereas in ripe soft peach fruit, the PME isoenzymes were solubilised by the same methods and, presumably, were more fully estimated.

6.5.3 Extraction Conditions and Substances Affecting PG Activity *in vitro*

The amount of enzyme activity extracted from a tissue depends on the interactions of the enzyme with the pectin/cell wall material. Interpretation of all experiments

requiring extracted plant enzymes must account for this serious limitation. In all oil palm tissues extracted at pH 5.0 (with 1M NaCl), it was found that PG still adhered strongly to the pectin. In the tomato, extraction conditions were reported to influence the major type of endo-PG isoform obtained, either PG1 or PG2 (Pressey, 1988). When fruit were extracted with 1M NaCl at pH 6, equal amounts of PG1 and PG2 were obtained, but when extracted with water at pH 1.6 only PG2 activity was obtained in the extract. Subsequent extraction of the pH 1.6 pellet yielded high levels of a protein called the ß-subunit. Other research (Pogson *et al*, 1991) showed that PG1 comprised the PG polypeptide and another protein called the ß-subunit. The use of transgenic tomato fruit expressing an antisense gene to the ß-subunit protein has shown that this protein influences pectin metabolism (Watson *et al*, 1994; Chun & Huber, 1997). Although it is still unresolved whether PG1 is an artefact of extraction (Pogson & Brady, 1993; Moore & Bennett, 1994), the increased susceptibility of cell walls to PG2 hydrolysis in ß-subunit deficient fruit (compared with wild type) implies a role for PG1 *in vivo* (Chun & Huber, 1997). It is interesting that the pectin-rich abscission zone of the oil palm, a monocotyledon, has similar basic PG isoforms to those found in the tomato pericarp. In addition, the effect of extraction at pH 1.6 alters the ratio of PG2 to PG1 (observed on IEF gels) as it does in the tomato; more pI 9.3 (PG2) enzyme and less pI ~9.0 (PG1) enzyme was observed (Fig. 6.10G). Complete cell separation occurring in the oil palm abscission zone is probably due to the induction of the exo-PG isoenzymes (pI 6.2-6.4), also shown to have high activity.

In the course of the experiments to determine PG activity in oil palm tissues, it was noted that di- and tricarboxylic acids were inhibitory. Tables 6.8-6.10 show that EDTA, citrate, malate, malonate, α-ketoglutarate, oxalate and ampholines (polyamino/carboxylic acids) were all inhibitory to PG activity in separated zone extracts. Whilst the pI 6.2-6.4 PG isoenzymes (exo-acting) retained activity in 20mM triethanolamine/HCl pH 7.7, almost no activity for the basic isoforms (endo-activity) was obtained. PG activity was not obtained at high pH (pI 7.5-9.5) after fractionation on IEF gels. Others, too, have noted similar effects on the activity of PG. In experiments to determine the optimum extraction conditions for PG (from the alcohol insoluble solids of ripe pericarp tissue), Jackman *et al* (1995) reported that both EDTA and citrate reduced tomato PG activity. Nogata *et al* (1993) also found that EDTA and citrate inhibited the PG from ripe strawberry fruit. Ali

& Brady (1982) noted that prolonged exposure of tomato extracts to pH values above 7 resulted in substantial loss of PG activity.

Sodium azide was observed to have an unusual effect on oil palm PG activity. Azide is an inhibitor of respiration and is used to prevent bacterial growth. Since some oil palm extracts or fractions with low PG activity required long incubation times (up to 24h) azide was included in these assays. The effect of azide on PG activity was, therefore, investigated. Surprisingly, at low pH (3.3-4.0) azide (0.2-0.4% in the assay) increases PG activity (in extracts of separated zone) by nearly 3-fold, but at a higher pH (5.3-6.0) although still acidic, the effect of azide is inhibitory, as shown below.

pH and % Azide in 100mM Acetate Buffer		**PG ACTIVITY nmol g Fw^{-1} min^{-1}**
pH 3.3,	0.0%	32.5
	0.4%	90.1
pH 5.3,	0.0%	106.7
	0.4%	47.7

Others, too, have reported the unexpected effects of azide; Bruinsma *et al* (1989) noted that "sodium azide and merthiolate added to media to prevent microbial contamination.....were found to interfere with PG-activity" in extracts of tomato. More recently, Uheda *et al*, (1995) reported that sodium azide caused the rapid abscission of branches of a small water fern, *Azolla*. They observed by light and electron microscopy that sodium azide induced the rapid dissolution (within 5-20min) of the middle lamella between cells, as well as modification of the primary wall of these cells. These events appeared to weaken both the cell walls and the adhesion between the cells causing cell expansion and rounding facilitating subsequent separation of the branchlet. Perhaps the increased PG activity in the presence of azide observed in the oil palm may also have been the reason for the cell separation observed in *Azolla* branches.

PG activity at low pH in separated zone tissue of oil palm can be increased in the presence of azide to near-maximum values obtained at pH 5.3. How is azide causing this effect? The molecule (N =N =N) is linear and can act like a halide ion. Pressey & Avants (1973) and others have shown that the activity of tomato PG1 and PG2 is increased in the presence of 0.1-0.25M NaCl. This has been suggested to be due to an ionic effect

disrupting hydrogen bonding in the pectin (Pressey & Avants, 1973) or salt stimulation of activity interacting with a salting out of substrate (Ali & Brady, 1982). All these results suggest that PG activity is particularly sensitive to charged groups (especially those which are negatively charged) not only in the oil palm abscission zone but also in tissues of other species, e.g. tomato pericarp. It is probably unlikely that halide ions result in PG activation *in situ* but the apoplast is a negatively charged environment and this complex ionic microenvironment of the cell wall and middle lamella no doubt modulates, regulates and exerts a control on PG activity *in vivo*.

6.5.4 Are the Polygalacturonase Isoenzymes in Oil Palm Similar to those in Other Fruits?

The activities of the pectic enzymes, polygalacturonase and pectin methylesterase, during the ripening of many fruits (mainly dicotyledons) have been extensively researched in the past 30 years. It is, therefore, relevant to compare this information with that obtained for the monocotyledonous oil palm fruit.

PG activity increases during ripening of fruits, such as the tomato (Hobson, 1963), apple (Wu *et al*, 1993), peach (Downs *et al*, 1990), papaya (Lazan *et al*, 1995), pear (Pressey & Avants, 1976) and avocado (Awad & Young, 1979). Two exo-PG enzymes and an endo-enzyme have been reported in ripe peach flesh (Downs *et al*, 1990). They were eluted by cation exchange chromatography with 50-100mM NaCl with the endo-enzyme eluting after the exo-enzymes. Similarly, ripe strawberry fruit contained an endo- and two exo-PG enzymes (Nogata *et al*, 1993). The PG enzyme(s) detected in oil palm mesocarp tissue was only the basic high pI isoform(s) (endo-acting, Fig. 6.2). Exo-acting enzymes (the pI 6.2-6.4 isoenzymes found in separated abscission zone tissue) were **not** present in the mesocarp. PG activity in the mesocarp is very low with no increase in activity during ripening and the fruit do not soften appreciably.

In abscission zone tissue the PG isoforms found in the oil palm appear to have some similarities with those reported for the peach fruit abscission zone (Bonghi *et al*, 1992) which contained two exo-enzymes (pI 5.8) and one endo-enzyme (pI 8.0). As discussed above, the oil palm endo-PG isoenzymes appear to be very similar to those of tomato fruit. The abscission zone PG also was recognised by antiserum to tomato fruit endo-PG (Fig. 6.11).

These results suggest that the isoforms of PG appear to be conserved proteins. Hydrolysis of the middle lamella and pectin matrix by PG may thus be similar throughout different species and tissue types. However, qualitative and quantitative differences in the PG isoforms can be found in fruit and abscission zones. In ripe fruit tissue, the amount of PG enzyme synthesized can be high (tomato pericarp) or low (oil palm mesocarp, apple). There also may be differences in the ratio of endo- to exo- PG isoforms in specific tissues (tomato, endo- > exo-; apple and oil palm, endo- only). In abscission zones of fruit such as peach and oil palm, both endo- and exo-PG isoenzymes are present, whereas in leaf abscission zones (lupin, bean), PG activity is very low and possibly due to exo-activity only (Berger & Reid, 1979; Henderson & Osborne, unpublished results).

CHAPTER 7

ß-1,3-GLUCANHYDROLASE - LAMINARINASE

Callose contains a significant proportion of ß-1,3-glucosidic linkages (confirmed in recent years by immunological techniques). In higher plants, callose is present in sclerenchyma, tracheids, xylem, phloem, fibre walls, parenchyma, plasmodesmata and many other tissues (Stone & Clarke, 1992). Other ß-1,3-glucosidic linkages occur in the cell walls and endosperm tissue of the monocotyledonous family, Poaceae (the grasses) as a mixed linkage ß-1,3:ß-1,4-glucan. ß-1,3-glucanhydrolases are found in extracts of most plant tissues, often with high levels of activity and since little is known about the callose or ß-1,3-glucan content in oil palm tissues, the ß-1,3-glucanhydrolase activity was investigated. Laminarin was used as substrate in these assays and, thus, the enzyme is referred to as "laminarinase" in this chapter.

Initially, the laminarinase activity was examined in ammonium sulphate protein preparations (Section 2.4), and was found to be highest in the cytosolic fractions of the separated zone and mesocarp in ripe fruit.

TISSUE	LAMINARINASE ACTIVITY nmol reducing groups g Fw^{-1} min^{-1}			
	UNRIPE UNSEPARATED		RIPE SEPARATED	
	Soluble	Cell Wall	Soluble	Cell Wall
Abscission zone	30.0	10.0	330.0	46.7
Mesocarp	58.3	13.3	138.3	23.5
Pedicel	31.7	5.0	10.2	13.3

Table 7.1: **Laminarinase Activity in Ammonium Sulphate Protein Preparations of the Abscission Zone, Mesocarp and Pedicel Tissues of Unripe Unseparated and Ripe Separated Fruit (tissue samples collected 48-72h post-harvest)**

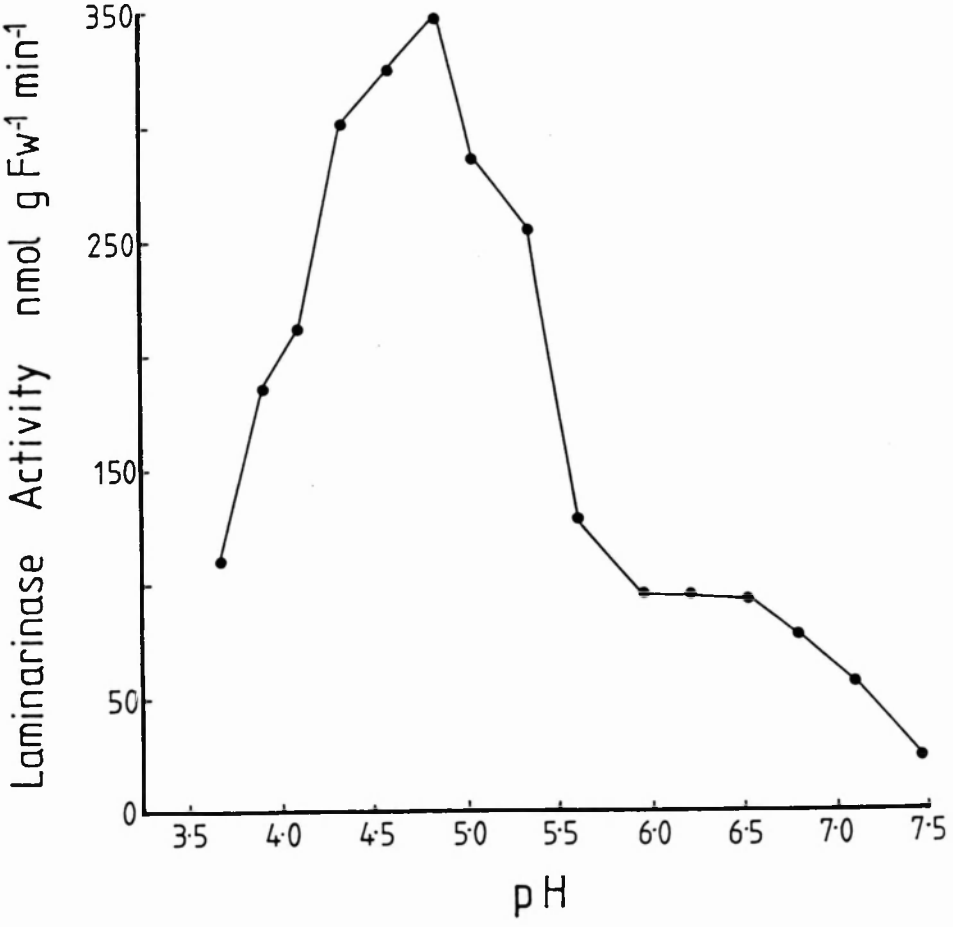


FIG. 7.1: THE PH-DEPENDENT LAMINARINASE ACTIVITY

The laminarinase activity of an enzyme extract of separated zone (150 daa) (determined as described in Section 2.6.2) was examined between pH 3.7-5.6 in 100mM acetate buffer and between pH 5.9-7.4 100mM phosphate buffer.

7.1 CHARACTERIZATION OF OIL PALM LAMINARINASE

7.1.1 Sequential Extraction of Separated Zone Tissue

Zone tissue (FZ) of ripe fruit separated at 24h after arrival was sequentially extracted with water (x2) then with 250mM acetate buffer pH 5.0 including 1M NaCl (x2); and the laminarinase activity in each of these extracts is shown in Table 7.2.

EXTRACTANT	Laminarinase Activity nmol g Fw^{-1} min^{-1}
1. H_2O - 1st extraction	444.8
2. H_2O - Extraction of pellet from 1 above	100.7
3. 250mM NaOAc/1M NaCl - Extraction of pellet from 2 above	66.9
4. 250mM NaOAc/1M NaCl - Extraction of pellet from 3 above	31.0

Table 7.2: Laminarinase Activity in the Sequential Extracts of Separated Zone Tissue

The extraction with buffered 1M NaCl does not solubilise additional laminarinase enzyme; the majority of activity is obtained in the first water extracts (85%).

7.1.2 The pH-Dependent Activity of Laminarinase

Separated zone (FZ/PZ) tissue was used for determining the pH optimum of the enzyme (as described in Sections 2.4.1 and 2.6.2). Acetate and phosphate buffers (both at 100mM) were used for these determinations; 100mM acetate at pH 4.8 was optimal (Fig. 7.1). At higher pHs activity was greatly reduced in phosphate buffer and also in Tris/HCl buffer (not shown). Therefore, pH 4.8 acetate buffer was used in all further experiments for laminarinase activity.

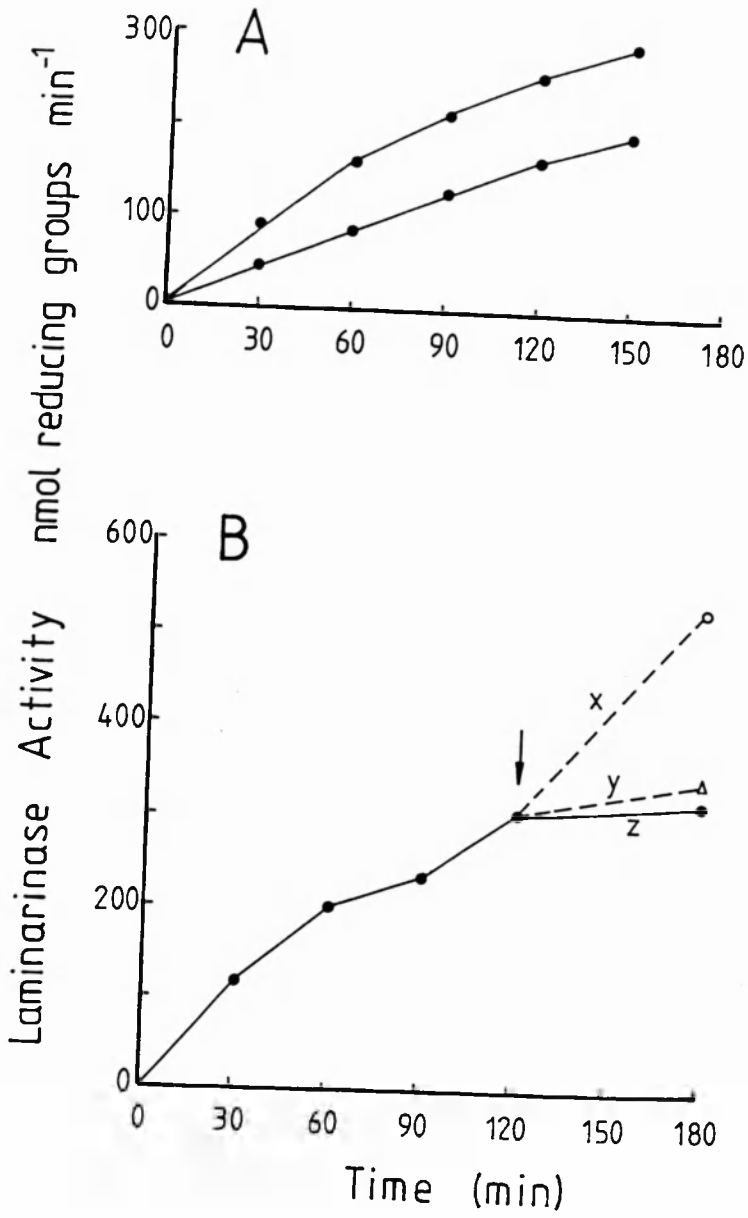


FIG. 7.2: THE TIME COURSE OF LAMINARIN HYDROLYSIS (1.5mg in each assay)

A. An extract of tepal base 1. Linearity is maintained for 2h when activity is low, but with twice the activity linearity in only just maintained for 1h.

B. An extract of ripe separated zone. After 2h, 30μl of fresh substrate (30mg/ml) was added to the assay tubes "x"; 30μl enzyme solution was added to the assay tubes "y"; and no addition to the assay tubes "z". The reaction continued for a further hour. The addition of fresh substrate restores linearity, but not the addition of more enzyme extract.

7.1.3 Time Course of Laminarin Hydrolysis

A substrate concentration of laminarin (10mg ml^{-1}) resulted in a non-linear assay in 1-2h incubations, but increasing the substrate concentration to 30mg ml^{-1} (30μl or 0.9mg in the 200μl assay solution) prevented this substrate limitation in 1h assays (Fig. 7.2A). In very active enzyme preparations (>200 nmol reducing groups formed per hour (Fig. 7.2A), the substrate concentration required to retain linearity was even higher. Therefore, to ensure linear rates in the samples, 50μl of 30mg ml^{-1} was used (1.5mg substrate in the 200 μl assay solution). If assays exceeded 100nmol reducing groups formed per hour, they were re-assayed for either a shorter time or with less enzyme extract. Figure 7.2B shows that linearity of activity was not restored when more enzyme extract was added to the reaction mixture ('y' in Fig. 7.2B), but it was on addition of fresh substrate ('x' in Fig. 7.2B).

The non-linearity of the time course of hydrolysis in enzyme extracts with high laminarinase activity suggests that the enzyme is endo-acting and that there may be only specific ß-1,3 linkages which can be hydrolysed. Since these extracts hydrolyse the laminarin substrate and form reducing groups that are readily detected (with TTC) in activity gels, it is possible that they may include some exo-ß-glucosidase activity. However, exo-activity can be measured by *p*-nitrophenol liberated from *p*-nitrophenyl-ß-glucopyranoside (ß-1,3, ß-1,4 and ß-1,6 exo-activity) and this was shown to be very low in separated zone (<75nmol g Fw^{-1} min^{-1}), mesocarp (<40nmol g Fw^{-1} min^{-1}) and tepal bases (<5nmol g Fw^{-1} min^{-1}) (compare Tables 8.3 and 8.4, page 188). The major part of the laminarinase activity assayed must therefore result from an endo-cleavage of the laminarin substrate.

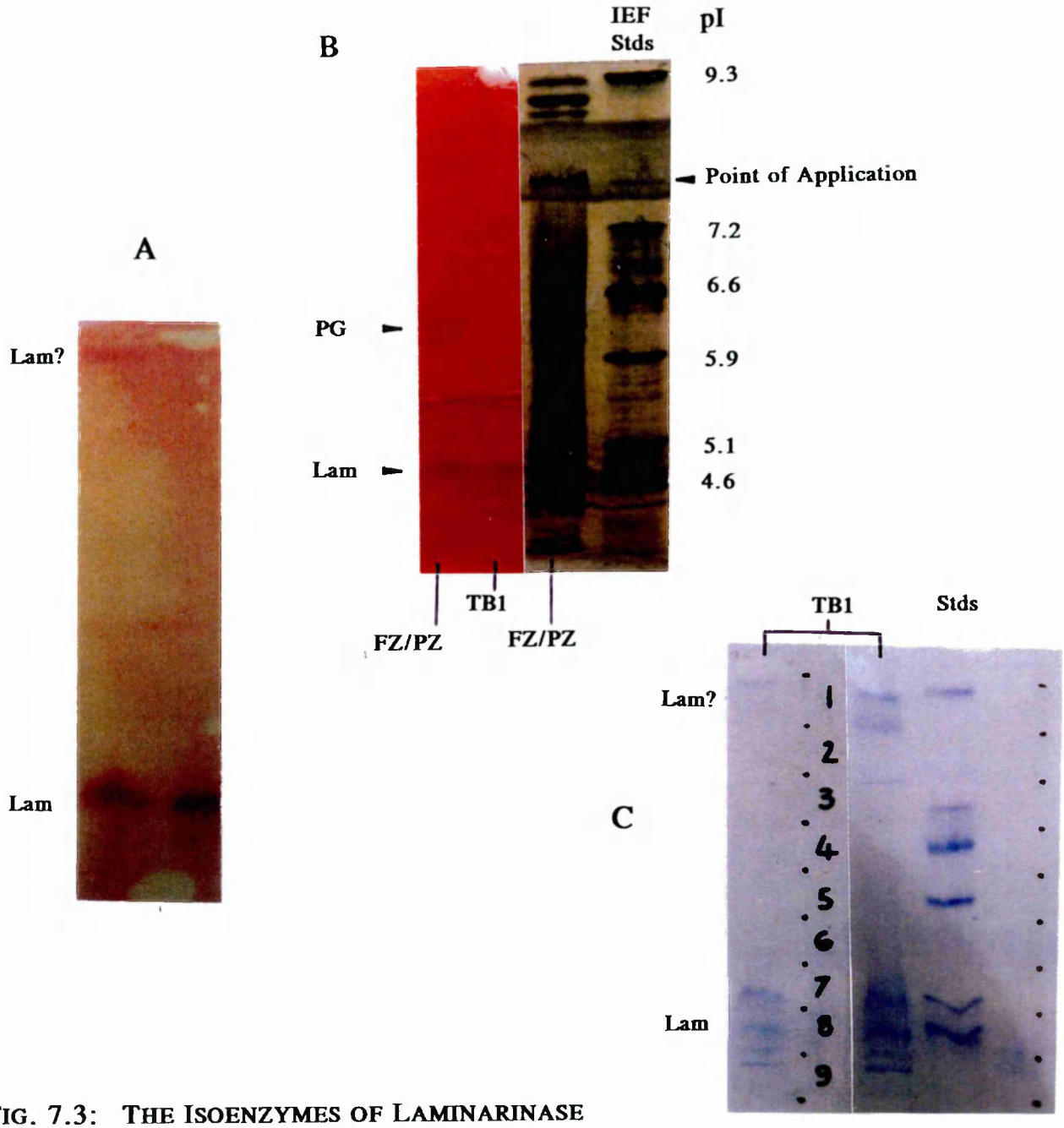


FIG. 7.3: THE ISOENZYMES OF LAMINARINASE

A. Extracts of separated zone FZ/PZ or tepal base 1 fractionated by IEF. After incubation with an overlay of laminarin substrate, the gels were stained for activity (TTC/NaOH). A major band of activity is present at acidic pI in both FZ/PZ and tepal base 1. Possibly some activity is present at pI 9.5 in FZ/PZ.

B. The pI of laminarinase was determined with an IEF standard protein mix and the pI 6.2-6.4 polygalacturonase isoenzymes in separated zone. Extracts of FZ/PZ, tepal base 1 and the IEF standard protein mix were fractionated by IEF. One half of the gel (FZ/PZ & TB1) was stained for laminarinase and polygalacturonase activity and the other half (FZ/PZ and IEF standards) was silver-stained for protein. The PG isoenzymes in activity and silver-stained gels were aligned and the approximate pI of laminarinase was between pI 4.6 and 5.1.

C. The pI of laminarinase was confirmed by a direct assay of gel slices. An extract of TB1 and the IEF standard protein mix was fractionated by IEF. The IEF standard proteins and two lanes of TB1 were stained with Coomassie Brilliant Blue for protein. The rest of the gel was equilibrated in buffer to pH 4.8 and then cut into 9 1cm sections. Each gel slice was extracted and the supernatant assayed for laminarinase activity which was detected in slice 8 between pI 4.6-5.1 (30.6nmol/h) and possibly slice 1 at pI 9.5 (1.5nmol/h).

7.1.4 Isoelectric Focusing and Ion Exchange Chromatography of Laminarinase

Concentrated extracts of separated zone and tepal base 1 were fractionated by isoelectric focusing (described in Sections 2.7 and 2.7.1). After removal of ampholytes, the gel was incubated with an overlay of laminarin substrate. Detection of the reducing group end-product with TTC (which gives a dark red precipitate) visualises the regions of laminarinase activity. Figure 7.3A shows only one prominent band of activity at acidic pI is present in both separated zone and tepal base 1, with the possibility of slight activity at about pI 9.5.

To determine the pI of the major band of laminarinase activity, an extract of separated zone (which also contains the pI 6.2-6.4 polygalacturonase isoenzymes) and tepal base 1 were fractionated by IEF along with an IEF standard protein mix. The gel was then cut in two and one half (separated zone and tepal base 1) was stained for both polygalacturonase and laminarinase activity. The other half (separated zone and IEF protein standards) was silver-stained for protein. Figure 7.3B shows a photograph of these gels; the silver-stained PG isoenzymes were aligned with those stained (TTC) on the activity gel. The laminarinase activity (also stained with TTC) was between pI 4.6 and 5.1, probably ~pI 4.8, determined from the IEF standard proteins.

Laminarinase activity at this pI also was confirmed by cutting a gel into 9x1cm slices. Proteins were extracted from each gel slice in acetate buffer and assayed for activity. Figure 7.3C shows that activity (cyanoacetamide assay for reducing groups) was present between pI 4.6 and 5.1.

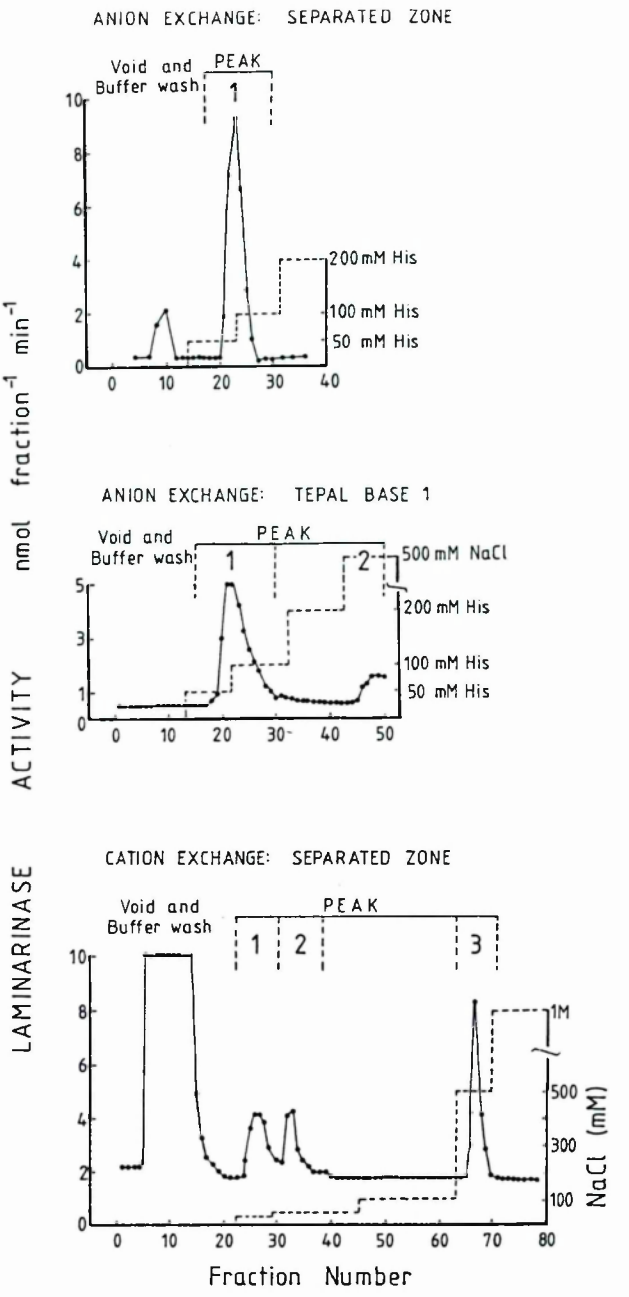


FIG. 7.4: ION EXCHANGE CHROMATOGRAPHY TO FRACTIONATE LAMINARINASE ISOENZYMES

A. An extract of separated zone (200mg fresh wt) was applied to the anion exchange support (Q-Sepharose) in 20mM triethanolamine buffer pH 7.7. Fractions were collected and assayed for laminarinase activity. A small peak of activity does not bind at pH 7.7 and is eluted in the buffer wash. This is possibly the pI 9.5 isoenzyme. The major peak (Peak 1) elutes when the pH is lowered to 5.0 with 50-100mM histidine buffer.

B. An extract of tepal base 1 (4g fresh wt) was applied to the anion exchange column as for (A) above. One major peak (Peak 1) of activity eluted when the pH was lowered to 5.0 with 50-100mM histidine buffer. A small peak (Peak 2) elutes with 500mM NaCl and since the control value (fraction alone; no added substrate) was the same as the test value, it was assumed to be due to the pectins/reducing groups in the extract.

C. An extract of separated zone was applied to a cation exchange column (Express-Ion C). The pectins in the extract did not bind at pH 5.5 and were eluted in the void and initial buffer wash. Three peaks of activity were then eluted with 25, 50 and 500mM NaCl.

When extracts of separated zone and tepal base 1 were fractionated by anion exchange chromatography (as described in Section 2.9), there was a major peak of activity which eluted when the pH was lowered from 7.7 to 5.0 (Peak 1, Fig. 7.4A,B). This peak corresponds with the enzyme visualised on IEF gels between pI 4.6-5.1. The pectin in the extract was adsorbed to the anion exchange support and eluted with 500mM NaCl (Peak 2, Fig. 7.4B). However, the laminarinase activity obtained after anion exchange chromatography was much lower than that found in the total extract. Since the extracts were initially desalted in 20mM triethanolamine at pH 7.7 and applied to the anion exchange column which was equilibrated in this buffer, it was possible that some activity was lost whilst at the higher pH.

When cation exchange chromatography at pH 5.5 was used (Fig. 7.4C), laminarinase activity did not bind to the column support because the laminarinase pI of approximately 4.8 was too low. As expected, therefore, most of the activity was washed off the column with 20mM acetate buffer. However, three small peaks of activity are eluted by a step-wise increase with buffered NaCl. The faint band of activity at pI 9.5 (Fig. 7.3A) observed on IEF activity gels may correspond to an activity in one or all of the peaks eluted (Peaks 1-3, Fig. 7.4C) and possibly represents another laminarinase isoenzyme.

7.1.5 Stability and Other Features of Oil Palm Laminarinase

At times it was necessary to prepare extracts prior to analysis, so the effect of freezing and thawing the same enzyme extract was investigated. Although it was found that enzyme extracts could be frozen and thawed several times without a decrease in activity, in practice the same extract was unfrozen only once.

TISSUE	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}		
	Before Freezing	Unfrozen x 1	Unfrozen x 2
Separated Zone - Normal	312.5	351.6	-
Separated Zone - Kluang Mutant	1245.3	-	1470.0
Mesocarp - Normal	216.8	-	244.8
Tepal Base 1 - Normal	268.5	-	291.8

Table 7.3: Laminarinase Activity in Thawed Frozen Enzyme Extracts of Separated Zone, Mesocarp and Tepal Base 1 Tissues.

In addition, frozen zone tissue (scraped cells) was thawed and left for 1-5d at 10°C or 1d at 23°C. Although this caused the tissue to brown, the activity of extracted laminarinase did not differ from that of the control tissue (extraction directly from the initial freezing).

SEPARATED ZONE (FZ)	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}
Control tissue (extraction directly from initial freezing)	919.4
Tissue at 10°C for 24h	953.8
Tissue at 10°C for 72h	910.0
Tissue at 10°C for 120h	991.7
Tissue at 23°C for 24h	994.5

Table 7.4: Laminarinase Activity in Separated Zone Tissue Thawed for 1-5 days at 10°C, and for 1 day at Room Temperature

The above treatments show that laminarinase is not a labile enzyme and that the conditions used in tissue collection, enzyme extraction and assay are sufficient to maintain extracted enzyme in its initial active state, ensuring valid comparisons between tissues and extracts prepared on different occasions.

Oil palm laminarinase showed no detectable activity (assayed by the increase in reducing groups) with lichenin substrate (ß-1,3 : ß-1,4-polyglucose). With another ß-1,3-polyglucose substrate (pachyman), several attempts were made to dissolve it with advice from Calbiochem but this proved impossible, so a suitable solution of this substrate could not be tested.

The approximate molecular weight of laminarinase is indicated by its behaviour during concentration in 10kD ultrafilters (Flowgen). About 42% of the activity passed through this filter (Table 2.3, page 51), which has a cut-off range of up to ~30kD (personal communication, Flowgen Instruments Ltd). Thus, it seems that the major isoform of laminarinase in oil palm fruit tissues has a MW of less than 30,000. It may be similar to that reported for ripening tomato (MW 12,000) (Wallner & Walker, 1975) and peach fruit (MW 19,000) (Hinton & Pressey, 1980).

7.2 LAMINARINASE ACTIVITY IN NORMAL AND MUTANT FRUIT TISSUES DURING RIPENING AND SEPARATION

7.2.1 Normal Fruit of Clone 271D

- **Mesocarp**

Laminarinase activity did not increase in the mesocarp as ripening progressed and no difference was found between the ripening and fully ripe tissues.

LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}	
RIPENING	**RIPE**
237.6 ± 40.2 n = 3 (194.2 - 273.7)	**200.8 ± 78.4** n = 7 (121.5 - 343.0)

Table 7.5: Laminarinase Activity in Enzyme Extracts of Ripening and Ripe Mesocarp

The ethylene production in the mesocarp of both ripening and ripe **unseparated** fruit was either below the level of detection or very low (~0.01nl C_2H_4 g Fw^{-1} h^{-1}). The laminarinase activity in ripening unseparated fruit was 237.6 ± 40.2nmol g Fw^{-1} min^{-1} and in ripe mesocarp from an unseparated fruit (156 daa), 227.5nmol g Fw^{-1} min^{-1}.

In the mesocarp of **separated** fruit, which were producing ethylene, the activity was 196.4 ± 84.9nmol g Fw^{-1} min^{-1} (n = 6). The mesocarp laminarinase did not show any increased activity immediately after the fruit began to produce ethylene at separation. However, the continued production of ethylene by the fruit mesocarp over many days may be responsible for further synthesis of the enzyme and its later greater activity (compare the Kluang mutant mesocarp, Section 7.2.2).

- **The Unseparated Zone**

The unseparated zone slice when excised from longitudinal sections of the fruit includes, in addition to the zone tissue, some mesocarp above the zone and pedicel below it. These tissues must, therefore, contribute to the total laminarinase value obtained. Since activity in the mesocarp was relatively high (Table 7.5), the actual activity in the unseparated zone may have been lower than the values shown in Table 7.6.

	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}	
UNSEPARATED ZONE	**RIPENING**	**RIPE**
ON ARRIVAL, 0h	**61.5 ± 11.0** n = 5 (44.8 - 70.8)	**86.5 ± 21.7** n = 3 (63.7 - 107.0)
48h AFTER ARRIVAL	**84.2 ± 26.1** n = 4 (46.5 - 105.3)	**84.2 ± 22.9** n = 3 (61.0 - 106.8)

Table 7.6: Laminarinase Activity in Enzyme Extracts of Unseparated Zone (Ripening and Ripe) on Arrival and 48h After Arrival in the Laboratory

The results show that activity was similar in ripening and ripe tissue and, even 48h after arrival (72h post-harvest), activity in the unseparated zone tissue had not increased. Thus, any stress-related increase in laminarinase due to harvesting or water deficit does not occur in the unseparated zone. This was also shown in a sample of very young unripe fruit (57 daa) which had not separated even after 168h (8 days post-harvest); the laminarinase activity was still low (115.0 nmol g Fw^{-1} min^{-1}).

- **Separated Zone**

Increased laminarinase activity in the zone occurred only at abscission in naturally shed ripe fruit, and in ripening and ripe fruit which separate after harvesting. The activity of FZ and PZ in fully ripe fruit shed in transit or on arrival in the laboratory was always 2-3x higher than in unseparated zones (compare Table 7.7 with Table 7.6).

SEPARATED ZONE	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}		
	FZ	PZ	FZ/PZ Combined
RIPE: Separated in transit or on arrival, 0h	**146.2 ± 50.0** n = 4 (96.3 - 214.3)	**318.0 ± 175.5** n = 4 (119.5 - 517.7)	**147.8 ± 36.7** n = 4 (117.7 - 198.8)
RIPENING: Separated, 48h after arrival	**196.6 ± 12.9** n = 2 (187.5 - 205.7)	**257.3 ± 17.7** n = 2 (244.8 - 269.8)	-
RIPE: Separated, 24-48h after arrival	**499.9 ± 119.8** n = 4 (363.0 - 643.3)	**319.9 ± 75.1** n = 2 (266.8 - 373.0)	**311.0 ± 67.8** n = 5 (217.0 - 377.7)

Table 7.7: **Laminarinase Activity in Enzyme Extracts of Ripe Separated Zone, the Fruit Side (FZ), Pedicel Side (PZ) and FZ/PZ Combined, on Arrival, and in Ripening and Ripe Separated Zone, 24-48h After Arrival in the Laboratory**

Separation (1+4/5) in one consignment of ripe fruit (147 daa) did not occur until 72h after arrival in the laboratory. The laminarinase activity in the FZ was 919.4 nmol g Fw^{-1} min^{-1} (the same sample as shown in Table 7.4). Such results suggest that laminarinase activity increases as separation occurs but the longer the process takes, the greater the increase in activity - possibly enhanced by the continuous ethylene production in the harvested fruit (but see Section 7.2.3).

- **Rudimentary Androecium**

The rudimentary androecium (RA) tissue was dissected from tepal tissue. No laminarinase activity was detected in the RA of unseparated ripening or ripe fruit. On separation, the activity remained low, 13.1 ± 11.5 nmol g Fw^{-1} min^{-1} (0.8-27.0 nmol g Fw^{-1} min^{-1}, n = 6). It cannot be excluded that this activity may have been due to small pieces of zone tissue which adhere to the base of the rudimentary androecium.

- **Tepal Base 1**

In **unseparated** fruit the laminarinase activity of tepal base 1 (the inner whorl of tepals adjacent to the fruit) was low; the activity of ripening and ripe fruit which had **not** separated, either on arrival in the laboratory or 24-48h after arrival, was not more than 99.3 nmol g Fw^{-1} min^{-1}, as shown in Table 7.8.

LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}	
RIPENING	**RIPE**
70.2 ± 15.4 n = 4 (49.0 - 85.7)	**53.8 ± 39.7** n = 3 (26.2 - 99.3)

Table 7.8: **Laminarinase Activity in Enzyme Extracts of Tepal Base 1 in Unseparated, Ripening and Ripe Fruit**

Before separation started at Position 1, laminarinase activity still remained low in tepal base 1 even 24h or 48h after arrival in the laboratory (26.2; 99.3 and 49.0; 75.3; 85.7nmol g Fw^{-1} min^{-1} respectively). This suggests that there is no increased activity as a result of post-harvest stress.

However, laminarinase activity in tepal base 1 increased with the progression of fruit abscission as separation of the tepal base from the pedicel proceeds. This is shown in Table 7.9 for ripe fruit at the different stages of fruit separation.

TEPAL BASE 1 OF RIPE NORMAL FRUIT	**LAMINARINASE ACTIVITY** nmol g Fw^{-1} min^{-1}
FRUIT NOT SEPARATED	**53.8 ± 39.7** n = 3 (26.2 - 99.3)
FRUIT STARTING TO SEPARATE AT POSITION 1 ONLY	**141.8 ± 41.0** n = 4 (93.7 - 181.0)
FRUIT SEPARATED 1+2/3 (on arrival, 0h) (Tepal 1 is attached to the pedicel, see Fig. 5.5B, page 119A)	**490.7 ± 101.5** n = 6 (369.7 - 616.8)
FRUIT SEPARATED 1+2/3 (24-48h after arrival) (Tepal 1 is attached to the pedicel, see Fig. 5.5B, page 119A)	**413.3 ± 157.0** n = 3 (317.3 - 594.5)

Table 7.9: **Laminarinase Activity in Enzyme Extracts of Tepal Base 1 of Ripe Fruit at Different Stages of Fruit Separation.**

Confirmation that laminarinase activity increases coincident with shedding is shown in fruit collected from the same fruit bunch but at different stages of separation. Natural abscission in the field is not synchronous and some ripe spikelets on arrival in the laboratory had fruit on them which were at three different stages of shedding: not

separated; separated only at Position 1; and shed 1+2/3. These fruit could be used to determine if the laminarinase activity increases as abscission progresses in the same sample, rather than a comparison of the different stages in different samples.

	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}		
TEPAL BASE 1	**Not Separated**	**Just Separating at Position 1**	**Separated 1+2/3**
Sample 1 (153 daa)	35.3	93.7	-
Sample 2 (153 daa)	99.3	170.4	317.3
Sample 3 (143 daa)	-	181.0	369.7

Table 7.10: Laminarinase Activity in Enzyme Extracts of Tepal Base 1 at the Different Stages of Zone Separation from three Separate Consignments of Fruit.

Once fruit started to separate, laminarinase activity began to increase and levels of about 500nmol g Fw^{-1} min^{-1} could be reached after the fruit had fully separated (1+2/3).

When tepal base 1 separated from the pedicel at Position 4/5, the activity still did not exceed ~500nmol g Fw^{-1} min^{-1}. Once induced, the laminarinase activity in tepal base 1 remained constant whether or not the tepal separateed from the pedicel (compare Table 7.9 and Table 7.11).

	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}	
TEPAL BASE 1	**RIPENING**	**RIPE**
SEPARATION 1+4/5 AFTER ARRIVAL (≥24h) (See Fig. 5.5D, page 119A)	**531.0** n = 1	**460.1 ± 33.8** n = 3 (434.1 - 498.3)

Table 7.11 Laminarinase Activity in Enzyme Extracts of Tepal Base 1 after Separation from the Pedicel at Position 4/5 (separation 1+4/5 occurs with the tepal whorls attached to the fruit).

One sample of tepal base 1 from ripe fruit (separated 1+4/5), collected after a much longer time (120h) still showed activity at a similar level (434.1nmol g Fw^{-1} min^{-1}), indicating the stable nature of the enzyme in stored material.

- **Tepal Base 2**

Tepal base 2 has attachment to the pedicel at Position 5 only and is not in direct contact with other tissues of the fruit. Assays of the laminarinase activity showed that they

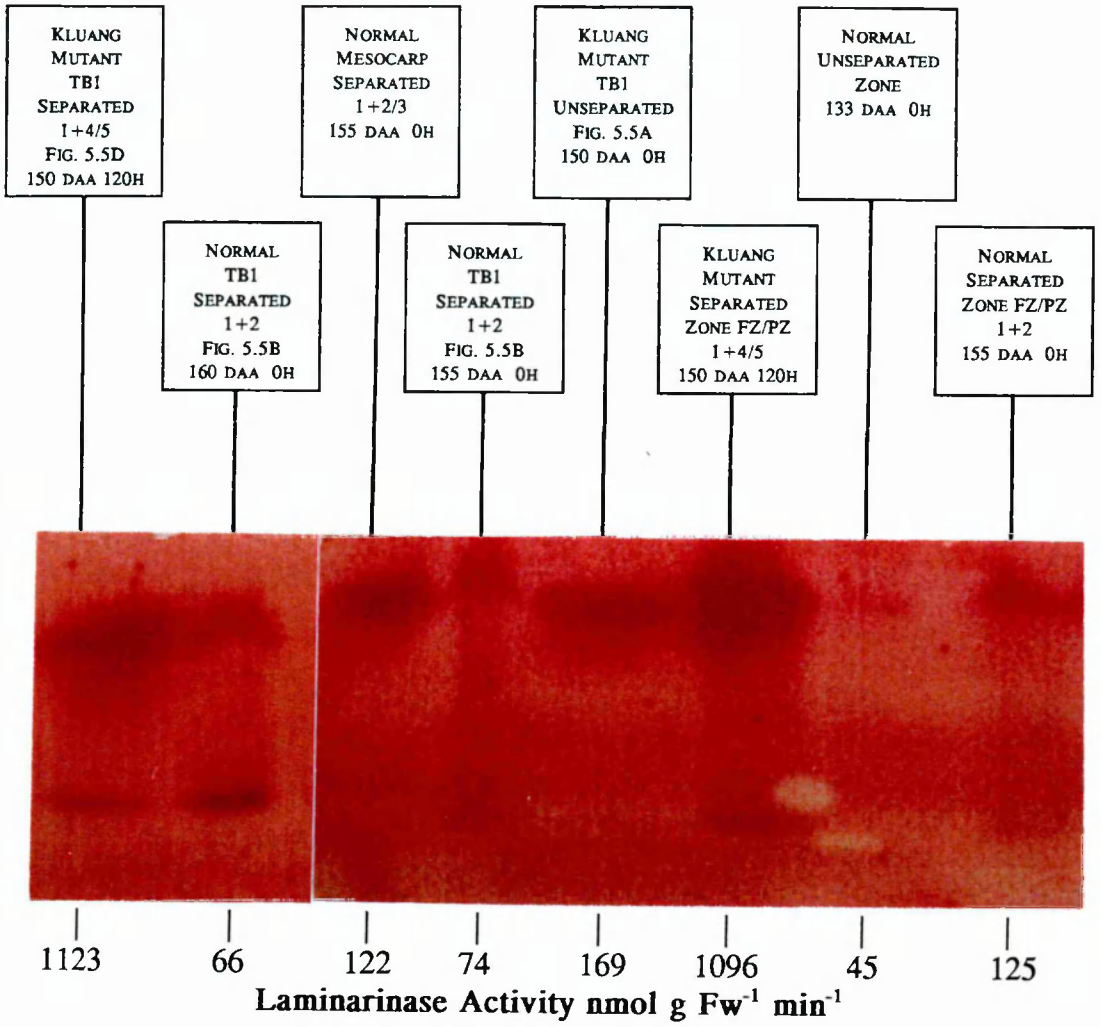


FIG. 7.5: THE ACTIVITY OF THE PI 4.8 LAMINARINASE ISOENZYME COMPARED IN THE NORMAL AND KLUANG MUTANT FRUIT TISSUES

Extracts were prepared with the same amount of tissue (1g for tepal base 1 (TB1), unseparated zone and mesocarp; 500mg for separated zone) and concentrated to the same volume (500μl) in a 3kD ultrafilter; 30μl of each concentrate was applied to an IEF gel (pI 3.5-9.5). The activity of each sample before concentration is shown.

After isoelectric focusing, the gel was equilibrated to pH 4.8 in 50mM acetate buffer (3 changes of buffer in 15min). It was incubated for 30min (30°C) an overlay of laminarin substrate, and then stained for reducing groups with TTC.

The stage of separation of tepal base 1 (TB1) samples is shown in Fig. 5.5 (page 119A).

were similar and very high, whether the tepal base had separated or was still attached to the pedicel (Table 7.12).

TEPAL BASE 2 OF RIPE FRUIT	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}
FRUIT SEPARATED 1+2 ON ARRIVAL, 0h (Tepal 2 is attached to the pedicel, see Fig. 5.5B, page 119A)	**557.0 ± 82.4** n = 2 (498.7 - 615.2)
FRUIT SEPARATED 1+3, 24h AFTER ARRIVAL (Tepal 2 is attached to the pedicel, see Fig. 5.5B, page 119A)	**442.5** n = 1
FRUIT SEPARATED 1+4/5, 48h AFTER ARRIVAL (Tepal 2 is attached to the fruit and separated from the pedicel, see Fig. 5.5D, page 119A)	**619.9 ± 47.9** n = 2 (586.0 - 653.7)

Table 7.12: Laminarinase Activity in Enzyme Extracts of Tepal Base 2 in Ripe Fruit Separated Either 1+2/3 (Tepal 2 attached to Pedicel) or 1+4/5 (Tepal 2 Separated from Pedicel).

7.2.2 Ripe Fruit of the Kluang Mutant

Laminarinase activity in the Kluang mutant was 2-3 times higher than in normal fruit in all the tissues examined. The higher activity of mutant fruit compared with normal fruit was also observed when extracts were fractionated by electrophoresis on IEF gels and when, after incubation with substrate, the presence of activity for laminarinase was visualised (with TTC) as shown in Fig. 7.5.

- **Mesocarp**

The laminarinase activity in ripe mesocarp was about twice that found in normal ripe mesocarp. This increased activity could be due to the higher ethylene production of the ripe mutant mesocarp (but see Section 7.2.3).

NORMAL	KLUANG MUTANT
200.8 ± 78.4 n = 7 (121.5-343.0)	**560.6 ± 169.1** n = 3 (385.8-723.3)

Table 7.13: Laminarinase Activity in Ripe Mesocarp Tissue of Normal and Kluang Mutant Fruit.

- **The Separated and Unseparated Zone**

Only a very few mutant fruit separate on arrival, and for all samples collected the majority of fruit separated with some pressure (1+3) in order to obtain sufficient tissue for extraction and assay. Although there was some variation between the samples of the separated zone analysed, the laminarinase levels were considerably higher than in normal separated zone (compare Table 7.7 with Table 7.14).

	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}		
TIME OF ZONE SEPARATION	**FZ**	**PZ**	**FZ/PZ Combined**
On arrival, 0h (184 daa)	866.7	1082.2	
24h after arrival (184 daa)	1157.8	1193.0	
48h after arrival (218 daa)	1723.0	2630.2	
48h after arrival (233 daa)	677.8	1153.5	
Mean ± Standard Deviation	**1106.3 ± 456.1**	**1514.7 ± 745.1**	
48h after arrival (221 daa)			485.9
120h after arrival (150 daa)			1096.3
144h after arrival (167 daa)			702.3
168h after arrival (195 daa)			462.6
Mean ± Standard Deviation			**686.8 ± 293.6**

Table 7.14: Laminarinase Activity in the Separated Zone of the Kluang Mutant, Fruit Side (FZ), Pedicel Side (PZ) and FZ/PZ Combined, at Various Times of Zone Separation.

Since laminarinase activity was so high in the mutant mesocarp, the actual activity in the unseparated zone (which contains mesocarp and pedicel tissues) was difficult to assess. Two samples of the ripe unseparated zone were analysed; the activity found was 233.7nmol g Fw^{-1} min^{-1} (167 daa) and 576.8nmol g Fw^{-1} min^{-1} (184 daa). These levels of activity were considerably higher than those found in the zones of comparable unseparated normal fruit (Table 7.6).

- **Tepal Base 1**

In normal fruit when Position 1 began to separate, even though no separation had occurred at the periphery of the fruit (Positions 2/3), laminarinase activity began to increase in tepal base 1 (Table 7.9). This suggests that the increase is a response to the initiation of separation at Position 1.

Kluang mutant fruit (after harvesting) are slow to separate but, unlike normal fruit, laminarinase activity increased in tepal base 1 even though separation had **not** occurred

(Table 7.15). In addition, the laminarinase activity reached very high levels, not obtained in normal tepal base 1, possibly because the post-harvest separation in the mutant is a prolonged event (Tables 7.15 and 7.16).

	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}	
TEPAL BASE 1	**NOT SEPARATED**	**SEPARATED**
218 daa, on arrival, 0h	212.2	749.7
194 daa, 120h after arrival	414.0	733.7
206 daa, 240h after arrival	567.0	862.3

Table 7.15: Laminarinase Activity in Tepal Base 1 of the Kluang Mutant. Spikelets with both Unseparated and Separated Fruit were used to collect the two Different Tepal Base 1 Samples.

TEPAL BASE 1 OF KLUANG MUTANT FRUIT	**LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}**
FRUIT **NOT** SEPARATED ON ARRIVAL (0h)	
153 daa	115.8
167 daa	131.7
180 daa	168.5
218 daa	212.2
218 daa	286.7
221 daa	67.3
223 daa	70.8
Mean ± Standard Deviation (n = 7)	**150.4 ± 79.1**
FRUIT **NOT** SEPARATED AFTER ARRIVAL	
153 daa (72h)	616.7
171 daa (48h)	376.2
171 daa (48h)	583.5
194 daa (120h)	414.0
206 daa (240h)	567.0
Mean ± Standard Deviation (n = 5)	**511.5 ± 108.6**
FRUIT **SEPARATED** 1+3 WITH PRESSURE	
153 daa (72h)	738.8
194 daa (120h)	733.7
218 daa (0h)	749.7
221 daa (48h)	571.2
Mean ± Standard Deviation (n = 4)	**698.4 ± 85.0**
FRUIT **SEPARATED** 1+4/5	
(Tepal base 1 attached to the fruit, see Fig. 5.5D, page 119A)	
150 daa (120h)	1457.3
153 daa (72h)	728.5
206 daa (240h)	862.3
218 daa (48h)	1358.8
Mean ± Standard Deviation (n = 4)	**1101.7 ± 360.2**

Table 7.16: Laminarinase Activity in Tepal Base 1 of the Kluang Mutant Fruit, Unseparated, Separated 1+3 or 1+4/5

Tepal base 1 tissue was examined in fruit from different positions in the bunch and at different stages of separation from one consignment of the Kluang mutant (218 daa) and the same pattern of high activity was confirmed (Table 7.17; compare with normal fruit in Table 7.10).

TEPAL BASE 1 (218 daa)	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}
Fruit not separated, mid-fruit (on arrival)	212.2
Fruit not separated, damaged terminal fruit (on arrival)	286.7
Fruit separated 1+2/3 with pressure (on arrival)	749.7
Tepal base 1 separated 1+4/5 (48h after arrival) (Tepals attached to the fruit after separation from pedicel)	1358.8

Table 7.17: Laminarinase Activity in Tepal Base 1 of Kluang Mutant Fruit of the Same Bunch (218 daa), Unseparated, Separated 1+2/3 or 1+4/5

7.2.3 Effect of Applied Ethylene on Laminarinase Activity

In normal fruit, laminarinase activity in tepal base 1 increased with the progression of zone separation (and ethylene production) until the fruit had fully separated. In the Kluang mutant, there was no separation in the fruit, and post-harvest separation was very slow, especially in unripe fruit. The effect of ethylene on laminarinase activity was therefore examined in tepal base 1 of unripe mutant fruit (153 daa). Fruit were incubated in either air + mercuric perchlorate (MP) or ethylene (10μl l^{-1}) for 72h (in 12 litre glass tanks with aeration every 24h). At 48h after arrival in the laboratory, the fruit were producing only very low levels of ethylene (<0.1nl C_2H_4 g Fw^{-1} h^{-1}) (Table 4.6, page 105). Tepal base 1 was collected from fruit which did not separate in the ethylene depleted air, and from those incubated in ethylene which separated 1+2/3 or 1+4/5. Laminarinase activity in tepal base 1 increased ~5-fold from the time of arrival, but rose to similar levels in both air and ethylene treated fruit by 72h. Unlike normal fruit, the level reached did not relate to the timing of separation or to whether the tepals remained attached to the pedicel (1+2/3) or were shed with the fruit (1+4/5), as shown in Table 7.18.

TEPAL BASE 1 (153 daa)	LAMINARINASE ACTIVITY nmol g Fw^{-1} min^{-1}
Fruit not separated, on arrival (0h)	115.8
Incubated in Air + MP for 72h: Fruit not separated	616.7
Incubated in C_2H_4 (10μl l^{-1}) for 72h: Fruit separated 1+2/3 (Tepals attached to the pedicel, Fig. 5.5B, page 119A)	738.8
Fruit shed 1+4/5 (Tepals attached to the fruit, Fig. 5.5D, page 119A)	728.5

Table 7.18: Laminarinase Activity in Tepal Base 1 of Kluang Mutant Fruit of the Same Bunch (153 daa), Unseparated and Separated in Ethylene after 72h Incubation

The majority of the fruit in air + MP eventually separated by 168h after arrival. Possibly laminarinase activity would have reached even higher levels by this time, as shown for the 218 daa sample (Table 7.17).

7.3 SUMMARY AND DISCUSSION

ß-1,3-glucanases are abundant proteins found in all higher plants examined (Stone & Clarke, 1992). There is evidence that they help defend plants against infection; ß-1,3-glucanase and chitinase (which hydrolyses N-acetyl glucosamine polymers in fungal cell walls) activities increase concomitantly in plants in response to ethylene, pathogen attack and pathogen-derived elicitors. They have been shown to act synergistically in the degradation of isolated fungal cell walls and to inhibit the *in vitro* growth of potentially phytopathogenic fungi (Mauch *et al*, 1992). The important role of ß-1,3-glucanase in pathogenesis (especially fungal pathogens) has been confirmed by antisense transformation (Beffa & Meins, 1996). Surprisingly, with viral pathogens the antisense plants showed an enhanced resistance. It was suggested that antisense transformation affected the metabolism of callose and its accumulation served as a physical barrier to contain the viruses at the site of infection. This indicates that ß-1,3-glucanase mediates the dissolution of callose.

However, ß-1,3-glucanase may have an important role in addition to the pathogen-related response. For example, ß-1,3-glucanase and chitinase have been found to accumulate in floral tissue during normal development (Neale *et al*, 1990) and ß-1,3-glucanase (but not chitinase) induction occurs during emergence of the radicle in seed germination (Leubner-Metzger *et al*, 1995). Whilst the function of ß-1,3-glucanase is

largely unknown, its endogenous substrate is thought to be callose. The biosynthesis of callose (by plasma membrane-bound enzyme complexes) is very sensitive to any perturbation such as wounding, physiological stress or infection. Therefore, ß-1,3-glucanase activity may be necessary in the regulation of callose dissolution/removal. In fact, the function of callose in plant cells and the control of its biosynthesis is, at present, the subject of active research (van Amstel & Kengen, 1996; Kudlicka & Brown, 1997).

The ß-1,3-glucanases have been assigned to several classes (Class I, II and III) based on their primary structure, and occur as acidic and basic isoforms. Keefe *et al* (1990) hypothesized that acidic isoforms are a secretory form and found in the intercellular space, whilst the basic enzymes are vacuolar. The activity of ß-1,3-glucanase or laminarinase appears to be sensitive to hormone levels. Ethylene induces ß-1,3-glucanase activity in bean leaves but only in specific cell types - the lower epidermal cells and along the vascular strands (Mauch *et al*, 1992). Also, Leubner-Metzger *et al* (1995) have suggested that, based on their research with germinating tobacco seeds, the rupture of a specific region of the endosperm depends on the ABA-sensitive induction of ß-1,3-glucanase. Auxin, too, stimulates the appearance of a ß-1,3-glucanase in pea epicotyls (Stone & Clarke, 1992 and references therein).

The results presented in this chapter on the ß-1,3-glucanase in the oil palm fruit (measured with the poly-ß-1,3-glucose algal substrate laminarin) show that the major isoenzyme form is acidic with perhaps a minor basic form (Fig. 7.3). It appears to be a soluble enzyme, rather than ionically bound to the cell wall (Table 7.2). Since exo-glucosidase activity (α and ß) was very low assayed with *p*-nitrophenylglucopyranoside (Chapter 8), the high laminarinase activity measured (reducing group assay) indicates that it does not hydrolyse in an exo-fashion. In addition, the time course experiments (Fig. 7.2) to determine the optimum substrate concentration to maintain linearity (for 1h) in the assay indicated that even with a very high substrate concentration (0.75%) in the assay, only relatively few bonds in the laminarin substrate were hydrolysed. The hydrolysis of laminarin by the oil palm enzyme was very similar to that shown for a ß-1,3-glucanase from cultured tobacco tissues (Felix & Meins, 1985) and they calculate that no more than ~10% of the glucose equivalents present in laminarin were hydrolysed.

In peach and tomato fruit, only a slight increase in laminarinase activity occurred during ripening (Hinton & Pressey, 1980), whereas in the oil palm mesocarp, no increased activity was observed (Table 7.5). Whilst the activity of the mesocarp did not change

during ripening, greatly increased activity was observed in abscission tissues. The activity in extracts of unseparated zone and tepal base 1 was at first very low but increased about ~3-5 fold after fruit separate. This increase was sometimes greater (up to 10-fold) in fruit which separated after arrival in the laboratory (24-48h or more later). Laminarinase activity also increased with the extent of separation at Position 1 (Table 7.10).

This rise in activity occurred simultaneously with the onset of ethylene production by the fruit and maximal laminarinase induction appears to be sensitive to quite low ethylene levels. We have shown previously (Henderson & Osborne, 1994) that applied ethylene to spikelets which have shed their fruit but not the tepals, does not result in the accelerated separation of the tepals. However, in the Kluang mutant, the mesocarp tissue does have a higher laminarinase activity than in normal mesocarp and this is perhaps due to a longer exposure to ethylene or, alternatively, the mesocarp tissue may require higher levels of ethylene to induce laminarinase.

The question arises, does endo-ß-1,3-glucanase have a role in oil palm fruit abscission? The considerable increase in activity in the zone and tepal bases (but not in the mesocarp) at the time of ethylene production and fruit abscission suggests a tissue specific induction but this in itself does not confirm a role in cell wall hydrolysis. In the non-abscinding Kluang mutant very high laminarinase activities were obtained, even in unseparated fruit, and this indicates that laminarinase activity alone does not determine separation in the absence of other enzyme activities, such as cellulase and polygalacturonase.

In the abscission of *Phaseolus vulgaris* explants ß-1,3-glucanase activity increases more than 10-fold in 72h and it was suggested that the hydrolase functioned in the removal of phloem callose (Abeles & Forrence, 1970). ß-1,3-glucanase has also been shown to be induced by its substrate. When muskmelon seedlings were placed in a laminarin solution there was an increase in ß-1,3-glucanase activty of at least 3-fold in hypocotyls and a larger increase in the roots (Stone & Clarke, 1992, references therein). The changes occurring during abscission may perturb the plasma membrane resulting in callose synthesis. This could then induce more ß-1,3-glucanase synthesis. In addition, plasmodesmata are ruptured during cell separation and must be subsequently sealed to maintain cellular integrity. Localised callose deposition between the plasma membrane and cell wall is believed to perform this sealing function (Roy *et al*, 1997) and ß-1,3-glucanase

may play a part in this. Van Amstel and Kengen (1976) observed the transient appearance of callose during the culture cycle of tobacco suspension cells and suggest that it may be involved in cell growth and act as a temporal stabiliser of the newly incorporated plasma membrane or to consolidate the direction of cellulose microfibril deposition. The transient appearance and disappearance of callose would thus require callose synthase and ß-1,3-glucanhydrolase activities. These activities may be important during abscission and function to stabilise the plasma membrane and/or the cell wall during the dissolution processes. There are reactions other than the hydrolysis of callose in which ß-1,3-glucanase may function. A laminarinase enzyme from soybean cell walls catalyzed an exo-transglycosylation reaction with [^{3}H-Glc] to form low molecular weight oligomers, although the equilibrium of the enzyme *in vitro* favoured hydrolysis (Fry, 1995, and references therein). Perhaps this is a reason why during time course measurements, activity of the oil palm enzyme very soon becomes non-linear. These reactions may serve to "soften" the tissue as has been reported in seed germination.

Defence mechanisms in plants involve a response to elicitor molecules and laminarinase activation is usually observed (for example, Patier *et al*, 1995). In normal developmental processes, endogenous signal molecules may also elicit laminarinase as part of the pathway to co-ordinate these changes. In recent years, attention has focused on the role of ß-1,3-glucanase activity in the physiological processes of the uninfected plant (such as the softening of endosperm tissue during seed germination). Abscission is also a highly regulated developmental process, in which ß-1,3-glucanase may have a specific role. For example, in abscission, a signal may elicit laminarinase in zone cells. The enzyme may then function in alterations of cell wall structure or perhaps produce a further signal (a short chain oligomer). In the Kluang mutant, laminarinase reaches very high levels which may be purely the result of its response to the continued production of ethylene by the unseparated fruit. However, it may also be that the action of laminarinase is necessary prior to that of cellulase and polygalacturonase, and since it appears that the abscission cellulase is "disabled" in the Kluang mutant, laminarinase continues to accumulate in the mutant tissue.

In conclusion, the results with normal and non-abscinding mutant fruit indicate that laminarinase increases in abscission zones at fruit separation, and in delayed separation very high levels of activity are attained. The role of laminarinase in the abscission process and its endogenous substrate would be subjects for further research.

CHAPTER 8

EXO-GLYCOSIDASE ACTIVITY WITH *p*-NITROPHENYL SUBSTRATES AND OTHER GLYCOHYDROLASES

The activity of polygalacturonase and cellulase have been extensively studied in dicotyledons. However, other enzymes may also be important in fruit ripening and abscission in the palm, a monocotyledon. Initial experiments indicated that the activities of α-D- and ß-D-xylopyranosidase, ß-D- and ß-L-fucopyranosidase and α-L-arabinofuranosidase were very low in extracts of unripe and ripe mesocarp and of unseparated and separated zone. In addition, α-mannosidase showed very high activity in all these tissues. Since there was no induced activity on ripening or shedding, these glycosidases were not examined further. Results for other exo-glycosidases, some of which are induced, are presented here. These include α-D- and ß-D-glucopyranosidase; ß-D-cellobiosidase; α-D- and ß-D-galactopyranosidase; α-L- and ß-L-arabinopyranosidase; α-D- and ß-D-xylopyranosidase; and ß-D-mannopyranosidase.

8.1 GLYCOSIDASE ACTIVITY IN MESOCARP, ZONE, TEPAL BASE 1 AND PEDICEL TISSUES

The ammonium sulphate protein preparations showed that, for ß-galactosidase, ß-mannosidase and ß-glucosidase, activity increased in mesocarp and zone with ripening and separation (Table 8.1). No activity was detected in rudimentary androecium or tepal base tissue (not shown).

	nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}			
	Unripe Unseparated		Ripe Separated	
	Soluble	Cell Wall	Soluble	Cell Wall
ZONE:				
ß-glucosidase	0.6	2.9	33.4	9.2
ß-galactosidase	27.8	27.7	611.3	36.8
ß-mannosidase	0.3	3.2	145.9	5.0
MESOCARP:				
ß-glucosidase	1.1	1.0	13.8	7.2
ß-galactosidase	0.0	0.9	111.9	64.9
ß-mannosidase	1.5	-	6.4	4.8

Table 8.1: Glycosidase Activity in Ammonium Sulphate Protein Preparations

The ß-galactosidase and ß-mannosidase activity of these ammonium sulphate protein preparations was lower than activities obtained when tissue was extracted and desalted (as described in Sections 2.4 and 2.5.1) and shown in Table 8.2.

	RIPE SEPARATED FRUIT nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}		
	RA/Tepal Base 1	Mesocarp	Zone FZ/PZ
α-Galactosidase	0.6	104.7	84.5
ß-Galactosidase	0.1	412.3	891.3
ß-Arabinopyranosidase	0.3	215.6	301.1
ß-Mannosidase	0.5	79.7	275.1

Table 8.2: Glycosidase Activity in Desalted Enzyme Extracts

However, it was also possible to assay glycosidase activity directly in extracts of tissue which had not been desalted on a Biogel column. The sodium chloride (1M NaCl in the extraction buffer) was diluted to between 80-200mM in the assay and did not interfere with glycosidase activity. It was necessary to remove the pectins and substances <6000 MWCO present in these extracts in order to carry out the cyanoacetamide assay of reducing groups, but none of these compounds gave a high background absorbance at 400nm and so did not interfere with the *p*-nitrophenol assay. Thus, the gel filtration (Biogel 10DG column) of enzyme extracts could be omitted. Most glycosidase activities obtained for tissues extracted without desalting were higher than both the ammonium sulphate protein preparations and the desalted enzyme extracts, but irrespective of the way the tissue was extracted, a similar relative pattern of glycosidase activity was obtained as in Tables 8.1 and 8.2.

Further determinations of ripening, unseparated fruit tissue are shown in Table 8.3 and for ripe separated tissue in Table 8.4. All these glycosidases in ripening unseparated tepal base 1 tissue had low activity but the adjoining pedicel fibres and zone tissues had relatively high activities, especially ß-galactosidase. This indicates that the tepal base 1 tissue was very different from the rest of the separating cells of the zone.

It was difficult to determine values for glycosidase activities in the tissue of unseparated zone, since a cut slice of this tissue included some mesocarp from above and pedicel from below the actual zone. The enzymes showing high induced activity with

ripening and abscission (~50% or more) in either mesocarp or zone are marked with an asterisk in Table 8.4, and of these the most spectacular was the 3-fold rise in ß-galactosidase activity in the separated zone (compare Table 8.4 with 8.3).

	RIPENING UNSEPARATED FRUIT nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}			
	Tepal Base 1	**Mesocarp**	**Pedicel**	**Zone**
α-Glucosidase	1.3	3.2	14.0	27.8
ß-Glucosidase	2.5	37.4	38.8	38.7
α-Galactosidase	5.8	84.7	267.6	216.2
ß-Galactosidase	5.5	458.0	801.9	479.2
α-Arabinopyranosidase	4.2	150.5	151.3	227.2
ß-Arabinopyranosidase	1.7	45.4	138.0	200.1
ß-Mannosidase	1.5	101.5	135.2	281.1

Table 8.3: Glycosidase Activities in Enzyme Extracts of Ripening Unseparated Fruit

	RIPE SEPARATED FRUIT nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}		
	Tepal Base 1	**Mesocarp**	**Zone FZ/PZ**
α-Glucosidase	3.6	4.7	29.0
ß-Glucosidase	4.3	37.0	74.5
ß-Cellobiosidase	-	6.2	1.8
α-Galactosidase	24.9	320.8 *	382.9 *
ß-Galactosidase	32.0	860.8 *	1790.0 *
α-Arabinopyranosidase	30.3	176.6	505.1 *
ß-Arabinopyranosidase	7.9	152.1 *	399.4 *
ß-Mannosidase	14.0	189.6	325.6

* = Enzymes which show a 50% or more increase in activity than those shown in Table 8.3.

Table 8.4: Glycosidase Activities in Enzyme Extracts of Ripe Separated Fruit

The activity of ß-glucosidase was analysed in more detail in mesocarp and zone because the exo-activity can act co-operatively with cellulase (endo-ß-1,4-glucanhydrolase). However, activity was always very low for ß-glucosidase and negligible for ß-cellobiosidase (removal of a di-ß-1,4-glucose unit from a ß-1,4-glucose polymer).

- **Comparison of Glycosidase activity in the Normal and Kluang Mutant Separated Zone**

Enzyme extracts were prepared and desalted as described in Sections 2.4.1 and 2.5.1. Activities were essentially similar in the separated zone tissue of the normal fruit and in the mutant fruit that separated in the laboratory, as shown in Table 8.5.

	RIPE SEPARATED FRUIT nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}	
	Normal Separated Zone	**Kluang Mutant Separated Zone**
ß-Cellobiosidase	1.2	0.9
α-Glucosidase	8.5	6.7
ß-Glucosidase	29.2	41.3
α-Galactosidase	72.1	44.1
ß-Galactosidase	735.2	704.9
α-Arabinopyranosidase	161.6	131.8
ß-Arabinopyranosidase	182.0	154.1
ß-Mannosidase	123.6	155.6
α-Xylosidase	2.9	0.8
ß-Xylosidase	18.0	14.7

Table 8.5: Glycosidase Activity in Normal and Kluang Mutant Ripe Separated Zone Tissue (desalted enzyme extracts)

The ß-galactosidase activity was examined again in direct tissue extracts of normal and mutant separated zone (Table 8.6). Results for the fruit side (FZ) and pedicel side (PZ) of the zone showed that ß-galactosidase activity was twice that in the desalted enzyme extracts (Table 8.5) and in both normal and Kluang mutant fruit, the activity extractable from the fruit side (FZ) was higher than from the pedicel side (PZ). In this sample, the activity in the Kluang mutant was somewhat higher than that in normal zone.

	ß-GALACTOSIDASE nmol *p*-NITROPHENOL g Fw^{-1} min^{-1}	
Separated Zone	**Normal (152 daa)**	**Kluang Mutant (212 daa)**
Fruit side (FZ) (n = 2)	1765 ± 36	2281 ± 20
Pedicel side (PZ) (n = 2)	1266 ± 11	1916 ± 5

Table 8.6: ß-Galactosidase Activity in Direct Extracts of Normal and Kluang Mutant Separated Zone Tissue at pH 3.7

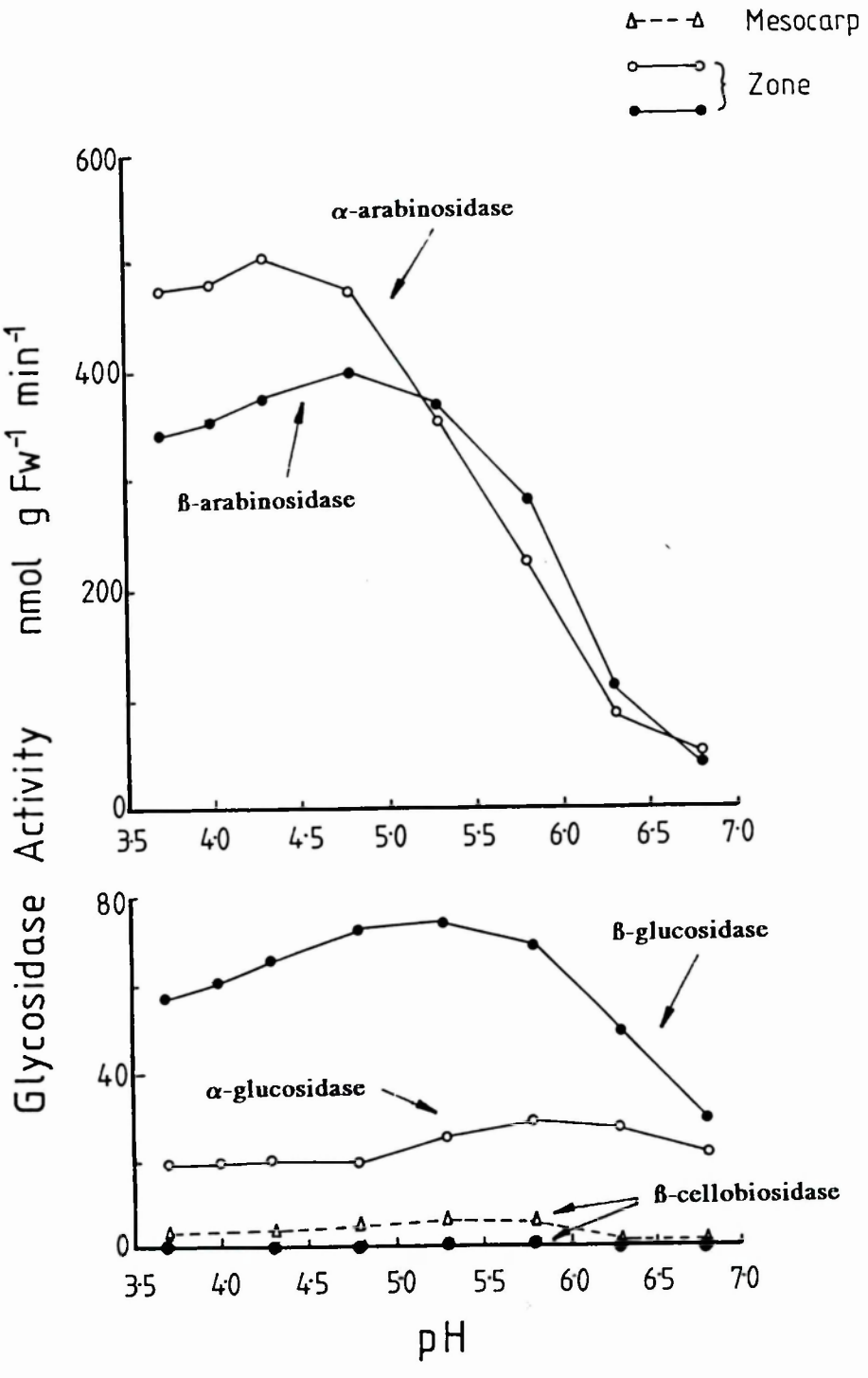


FIG. 8.1: GLYCOSIDASE ACTIVITY IN RIPE NORMAL FRUIT

Arabinosidase and Glucosidase Activity in Extracts of Separated Zone. Cellobioside Activity in both Separated Zone and Mesocarp.

Enzyme extracts of ripe tissues (152 daa) were prepared as described in Section 2.6.3 and assayed directly (without desalting). Activity was determined between pH 3.8-5.8 in 100mM acetate buffer and between pH 6.3-7.8 in 100mM phosphate buffer.

8.2 pH-DEPENDENT ACTIVITY OF THE GLYCOSIDASES

Initially, the pH-dependency of the glycosidases was examined in the buffers: glycine/HCl, acetate and citrate/phosphate between pH 2.4-5.0; acetate, citrate/phosphate, MES, TES and phosphate between pH 5.0-7.5. Highest activity was always obtained in acetate buffer and above pH 7.0 the activity of all glycosidases was very low. The glycosidase activities shown in Figs. 8.1 & 8.2 are the results of one experiment (in 100mM acetate pH 3.7-5.8 and 100mM phosphate pH 6.3-6.8) which has been repeated at least six times on different samples. Although the glycosidase activity in some of these assays has been lower overall due to the method of enzyme extraction and partial purification, the same relative pattern of activity was always obtained.

The pH activity profile of α-D- and ß-D-glucopyranosidase, α-L- and ß-L-arabinopyranosidase, α-D-galactopyranosidase and ß-D-mannopyranosidase was determined for separated zone FZ/PZ tissue and ß-cellobiosidase for mesocarp and separated zone. Since ß-galactosidase was very high at pH 3.7, a lower pH range between 2.4-3.6 (100mM glycine/HCl) was also analysed for both mesocarp and separated zone (Fig. 8.3).

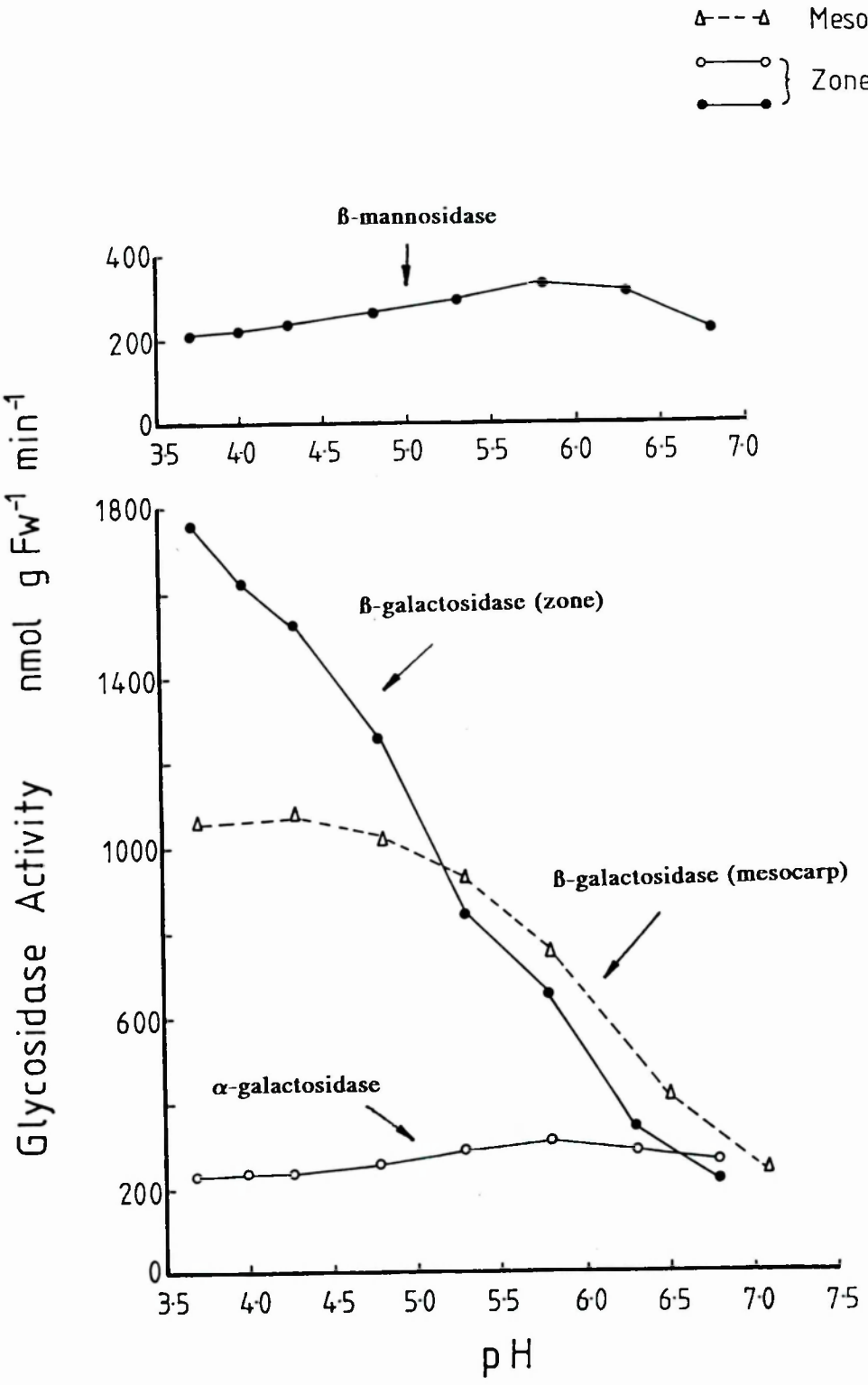


FIG. 8.2: GLYCOSIDASE ACTIVITY IN RIPE NORMAL FRUIT

ß-Mannosidase and α-Galactosidase Activity in Extracts of Separated Zone. ß-Galactosidase Activity in Extracts of Separated Zone and Mesocarp.

Enzyme extracts of ripe tissues (152 daa) were prepared as described in Section 2.6.3 and assayed directly (without desalting). Activity was determined between pH 3.8-5.8 in 100mM acetate buffer and between pH 6.3-7.8 in 100mM phosphate buffer.

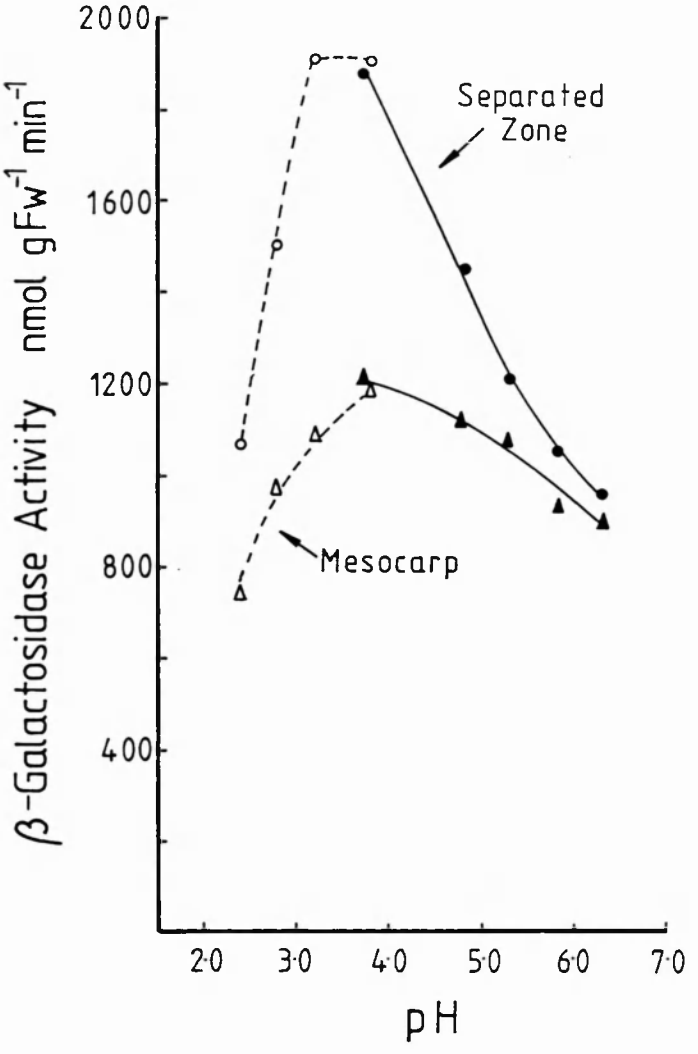
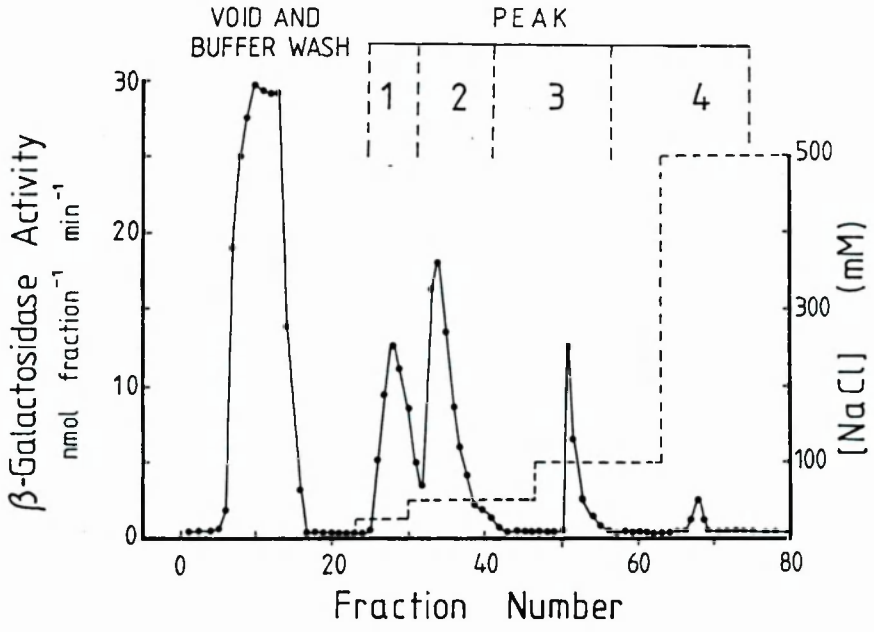


FIG. 8.3: THE PH-DEPENDENT ACTIVITY OF ß-GALACTOSIDASE BETWEEN PH 2.4-6.3

Enzyme extracts of ripe separated zone and mesocarp (152 daa) were assayed for ß-galactosidase activity between pH 2.4-6.3.

Broken line = 100mM Glycine/HCl buffer pH 2.4-3.8
Solid line = 100mM Acetate buffer pH 3.7-6.3

A



B

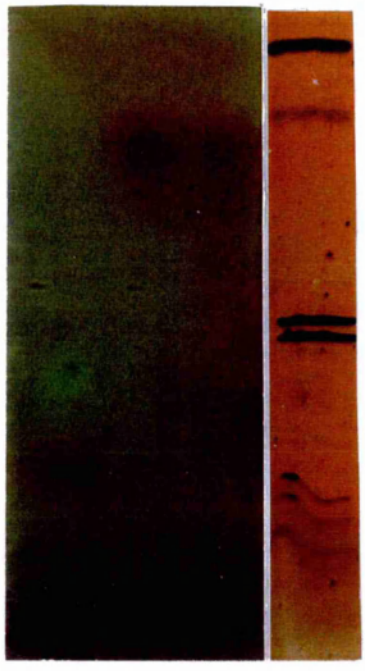


FIG. 8.4: ISOENZYMES OF ß-GALACTOSIDASE IN SEPARATED ZONE TISSUE

A. An enzyme extract was fractionated by cation exchange chromatography and eluted with step-wise increases in NaCl concentration.

B. Fractions from the 50mM, 100mM and 500mM NaCl elutions were pooled, concentrated and desalted (3kD ultrafilter). The concentrates (30μl) were then fractionated by isoelectric focusing. The gels were equilibrated to pH 3.7, incubated with an overlay of *p*-nitrophenyl-ß-galactopyranoside (2h, 30°C) and stained for activity (TTC). The pH 1.6 extract of separated zone (silver-stained for protein, see Fig. 6.10G) was fractionated on the same gel and aligned for comparison of pIs.

8.3 ISOENZYMES OF ß-GALACTOSIDASE IN THE ZONE

The fractions obtained by cation exchange chromatography (for the PG isoenzyme analysis) were also assayed for ß-galactosidase activity. There were four peaks of activity in ripe separated zone (Fig. 8.4A). The same peaks occurred in both unseparated and translucent zones (not shown) but with lower activities in all four peaks.

The 50mM, 100mM and 500mM NaCl eluted fractions from the cation exchange chromatography (which were pooled, concentrated and desalted for the PG isoenzyme analysis) were fractionated by isoelectric focusing and stained for activity with TTC (after incubation with *p*-nitrophenyl-ß-galactoside). Figure 8.4B shows that an acidic isoform pI ~5.1 is present in the 50mM NaCl eluted fractions and a basic isoenzyme pI ~8.6 in the 100mM and 500mM NaCl eluted fractions.

8.4 OTHER GLYCOHYDROLASES

8.4.1 Endo-ß-1,4-Mannanase

Pressey (1989) reported endo-ß-1,4-mannanase activity in extracts of ripe tomato fruit assayed by the decrease in viscosity of locust bean gum substrate. Locust bean gum is composed of a linear backbone of ß-1,4-linked mannose units with branches of single α1-6-linked galactose residues on every fourth mannose. The exo-glycosidase, α-galactosidase, will slowly hydrolyse the galactose side chains in galacto-mannose and leave end groups more susceptible to exo-ß-mannosidase.

For oil palm, ripe separated zone, mesocarp, and tepal base 1 extracts were examined for activity using a 1% locust bean gum solution at pH 4.5 (Section 2.6.1). Activity was barely detectable after 2.5h incubation (30°C).

Tissue	% Decrease in Viscosity at pH 4.5 (2.5h)
Separated zone FZ/PZ	2.0
Mesocarp	4.0
Tepal base 1	0.0

Table 8.7: Endo-ß-mannanase activity in extracts of separated zone, mesocarp and tepal base 1 estimated by change in viscosity of a solution of locust bean gum.

Both exo-α-galactosidase and ß-mannosidase showed some activity in both separated zone and mesocarp (Section 8.1) but the negligible decrease in viscosity in 2.5h suggests that there was very little endo-ß-1,4-mannanase activity in these extracts.

Ammonium sulphate protein preparations did, however, show some mannanase activity in ripe mesocarp and separated zone (at pH 4.5) when estimated by the cyanoacetamide reducing group assay, although this activity could also be due to α-galactosidase and ß-mannosidase.

	ACTIVITY WITH LOCUST BEAN GUM nmol/min/g fw			
	UNRIPE		RIPE	
	Soluble	Cell Wall	Soluble	Cell Wall
Zone	0.9	0.2	24.6	1.8
Mesocarp	2.5	0.8	27.7	1.2

Table 8.8: **Endo-ß-mannanase, ß-mannosidase and α-galactosidase activity in extracts of zone and mesocarp estimated by the cyanoacetamide reducing group assay (Gross, 1982).**

8.4.2 Hydrolysis of Gum Arabic

Gum arabic is a hemicellulose fraction containing a branched polymer of galactose, rhamnose, arabinose and glucuronic acid. Hydrolysis of this substrate was analysed by the cyanoacetamide reducing group assay (Section 2.6.2). The ammonium sulphate protein preparations of unripe and ripe zone, mesocarp and tepal bases showed no activity at all. Low activity was detected in separated zone (10.9 nmol reducing groups g Fw^{-1} min^{-1}). This activity also was probably due to exo-galactosidase and exo-arabinosidase (Section 8.1).

8.4.3 Xylanase

No activity in zone, mesocarp or tepal base tissue was detected with xylan substrates (birchwood and nasturtium seed). Enzyme extracts were incubated for up to 3h at pH 4.5 (either a viscosity assay or the cyanoacetamide assay for reducing groups). However, xylanase activity may have been detected with a longer assay, different pH and/or more enzyme extract.

8.5 SUMMARY AND DISCUSSION

The low activity with the *p*-nitrophenyl substrates of both ß-cellobiosidase and ß-glucosidase (exo-activity) indicate that the endo-ß-1,4-glucanase (cellulase) activity in oil palm is unlike the fungal and microbial cellulases where these enzymes act together in degrading cellulose. Exo-xylosidase activity was also very low, and possibly, too, endo-xylanase, suggesting that the action of these enzymes is not a major feature of ripening or abscission in oil palm fruit.

ß-Galactosidase in separated zone tissue (Position 1) showed the greatest increase in activity but in the separated tepal bases (Position 4/5) all the glycosidases examined had very low activities (Tables 8.2-8.4). The pH dependent activity of ß-galactosidase in separated zone had the highest activity pH 3.3-3.8 and at these pHs activity was always much greater than that in the mesocarp.

The glycosidase activity of the normal and Kluang mutant separated zone tissue (desalted enzyme extracts) was compared and no differences were detected. However, extracts assayed for ß-galactosidase without desalting (directly after homogenization and centrifugation), did have a higher activity in the Kluang mutant separated zone; about 1.3-1.5 times greater than that in the normal separated zone. But this does not implicate a determining role for ß-galactosidase in the non-abscinding condition of the Kluang palm.

Although the role of ß-galactosidases/ß-galactanases in fruit ripening is not known, Carey *et al* (1995) report that ß-galactanase activity in wild type tomato increased at the onset of ripening but no similar increase was detected in the non-ripening mutants *rin* and *nor*. Perhaps the induction or the increased activity of ß-galactosidase/ß-galactanase is dependent on the prior activity of other enzymes, such as cellulase, PME or PG.

CHAPTER 9

SIGNALLING MOLECULES IN ZONE SEPARATION

Research in our laboratory with *Phaseolus vulgaris* explants (Thompson & Osborne, 1994) indicated that signalling molecule(s) produced in the stele may subsequently initiate events leading to cell separation in cortical cells. The vascular tissue in the bean pulvinus is part of a central stelar core. With careful manipulation and micro-dissection, it is possible to remove the stele so that the sequence of cell separation events can be followed. It was found that no endo-ß-1,4-glucanhydrolase (cellulase, pI 9.5) is detected in the abscission zone cortex in de-steled explants, and no cell separation occurs. However, if the stele is removed after a lag period, but before any cellulase activity is present in the cortex, then cellulase (pI 9.5) is produced and cell separation ensues. Thus, the cortical cells of the abscission zone are able to separate independently of the vascular tissue only after the vascular tissue has begun to respond to abscission-promoting signals.

The vascular tissue in the oil palm abscission zone is not part of a central stelar core (as in the bean) but is distributed throughout the zone. This is shown in the phloroglucinol-HCl stained longitudinal sections of abscission zones in Fig. 3.10 (pages 88 and 88A). Although it was not possible to remove the vascular tissue, low molecular weight substances obtained from ripe naturally-separated zone tissue and then applied to tissue slices of unripe fruit could perhaps accelerate separation. Unripe young fruit (60-120 daa) will separate after harvesting, but it occurs very slowly, usually about 6 days later (ripening to ripe fruit usually separate within 48h after arrival in the laboratory).

To determine whether there was any abscission-promoting activity in separated zone, the scraped cells FZ/PZ tissue was extracted with water (1g of tissue + 2ml deionised water). After centrifugation, the supernatant was applied to a gel filtration column (Biogel 10DG) which had been calibrated with blue dextran/dichromate. Fractions containing the <6000 MWCO substances were collected and pooled. Longitudinal sections of the unripe abscission zone approximately 500-700μm thick were hand-cut with a new single-edge razor blade. A fixed volume pipette was used to apply 2μl of the

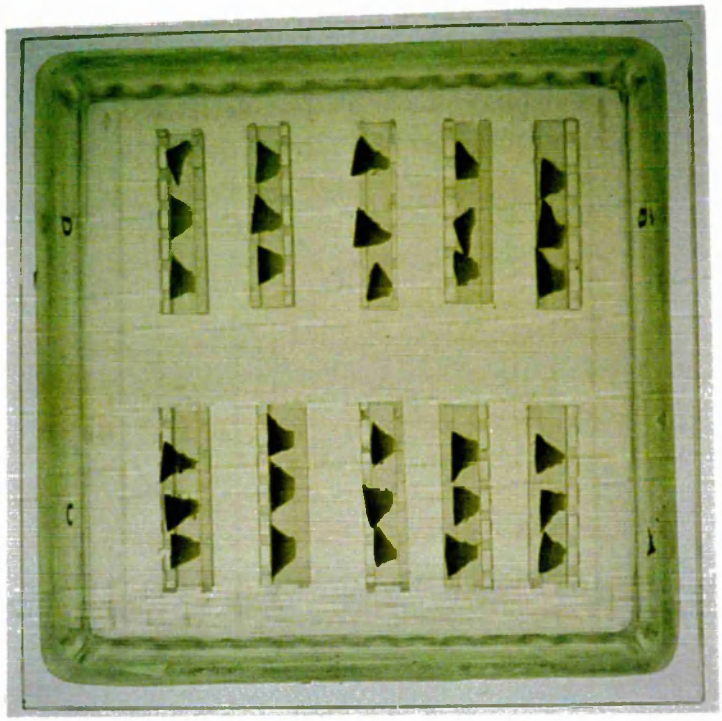


FIG. 9.1: EXPERIMENTAL PROCEDURE FOR THE DETERMINATION OF ABSCISSION PROMOTING SUBSTANCES IN SEPARATED ZONE EXTRACTS.

Hand-cut longitudinal fruit sections of the unseparated abscission zone of very young fruit (~60 daa) were placed on perspex racks over agar in a glass dish with lid. A solution of low molecular weight substances obtained from the separated zone extracts (2μl) was applied to the abscission zone. The dish was kept at 25°C in an incubator and the sections checked for separation after 24h and 48h.

< 6000 MWCO solution to the abscission zone. Since the abscission zone of the unripe fruit section is not easily visible (Fig. 3.6, page 82A) and the zone is not translucent at this stage, the section was held to the light. By transmitted light, the zone can be recognised so that the solution could then be applied to the zone area only. Control sections were treated with 2μl of water. Both the test solutions and the water controls contained chloramphenicol (10μg ml^{-1}) to prevent microbial contamination. The sections were then positioned on perspex racks which were in a glass dish containing 1-2% agar (to maintain humidity), and a glass lid was placed on the dish (Fig. 9.1). The sections were examined for the acceleration of zone separation compared with the controls at regular intervals up to 72h.

In the first experiment, solutions of ACC (1mM), the low molecular weight substances (< 6000 MWCO) from a water extract of ripe separated zone tissue and a water control were prepared with and without AOA (1mM), an inhibitor of ACC synthase. Each test consisted of 56 unseparated abscission zone slices of unripe fruit (57 daa). The results show that the application of the < 6000 low molecular weight substances from separated zone enhances separation approximating that of the ACC solution (Table 9.1).

	% SEPARATION IN 57 DAA FRUIT					
	+ AOA			- AOA		
Time	**ACC**	**H_2O**	**< 6000**	**ACC**	**H_2O**	**< 6000**
24h	26.8	1.8	25.0	58.9	28.6	41.1
48h	85.7	39.3	73.2	-	-	-

Table 9.1: **The effect of ACC, H_2O and the < 6000 MWCO solution (in the presence or absence of AOA) on zone separation in unripe fruit slice sections.**

The experiment was repeated with unripe (98 daa) and ripening (125 daa) tissue slices, without the inclusion of AOA (Table 9.2).

	% SEPARATION IN 98 DAA FRUIT			
	<6000		H_2O Control	
Time	Separating	Separated	Separating	Separated
41h	33.3	0.0	0.0	0.0
62h	33.3	41.7	16.7	16.7

	% SEPARATION IN 125 DAA FRUIT			
	<6000		H_2O Control	
Time	Separating	Separated	Separating	Separated
24h	57.7	3.8	9.5	4.8
48h	3.8	92.3	14.3	42.9

Table 9.2: **The effect of the <6,000 MWCO solution and a water control on unripe (98 daa) and ripening (125 daa) tissue slices.**

In order to isolate the abscission promoting substance(s), the <6,000 MWCO fractions of water extracted separated zone tissue (4g in ~30ml) was sent to Dr D. Ashford of the Glycobiology Laboratory, University of York. This sample was fractionated by reverse phase, anion and cation exchange chromatography. These partially purified fractions were tested on tissue slices as shown in Table 9.3.

	% SEPARATION IN 60 DAA FRUIT	
Fractionated Sample	46h	72h
REVERSE PHASE		
Unbound	0	5.0
Eluted	0	10.0
ANION EXCHANGE		
Unbound	0	20.0
Eluted	30.0	70.0
CATION EXCHANGE		
Unbound	25.0	35.0
Eluted	10.0	25.0
Control 1	0	0
Control 2	0	8.3

Table 9.3: **The effect of partially purified fractions of the <6,000 MWCO fractions on zone separation in unripe tissue slices (60 daa).**

The results of these experiments indicate the presence of an abscission-promoting substance that is negatively charged. However, more concentrated extracts are now required and further fractionation, concentration and testing of the low molecular weight signal substances from extracts of separated zones is currently continuing in collaboration with the Glycobiology Laboratory in the University of York.

CHAPTER 10

CONCLUSION

10.1 AIMS, OBJECTIVES AND OUTCOME OF THE RESEARCH

Our previous work (Appendix E) established that abscission in the monocotyledonous ripe oil palm fruit involved cell dissolution processes similar to those reported for other abscission systems (e.g. bean pulvinus) induced by ethylene. Natural abscission of oil palm fruit commenced firstly between the pedicel and fruit and after this, separation of the tepal bases from the pedicel occurred (Henderson & Osborne, 1990). The oil palm is one of the three fruits (oil palm, avocado and olive) which has an oil-bearing fruit mesocarp tissue (in contrast with oil-rich seeds). We examined the activity of the mesocarp lipase since information available suggested that this enzyme could be induced when maximum triacylglycerol synthesis had been reached. At this stage, it is possible that hydrolysis of fatty acids might act to signal full-ripeness, with concomitant ethylene production and abscission. However, surprisingly, our investigations showed that the lipase was induced early in ripening, and its increase paralleled the increase in the biosynthesis of TAG (Henderson & Osborne, 1991). In fact, the most important signal for abscission was found to be a surge in ethylene production which was independent of the activity of the lipase (Henderson & Osborne, 1994).

The aim of this present research was to establish the biochemistry of abscission at the different positions of separation with the objective of controlling fruit shedding on the plantation and achieving the goal of maximising oil yield. Naturally-occurring non-abscinding mutants were sought on Unilever plantations. One palm, the Kluang mutant, was investigated in detail and compared with normal clonal material (271D). The relative importance of specific enzymes in abscission was determined and a sequence or model of biochemical events is postulated.

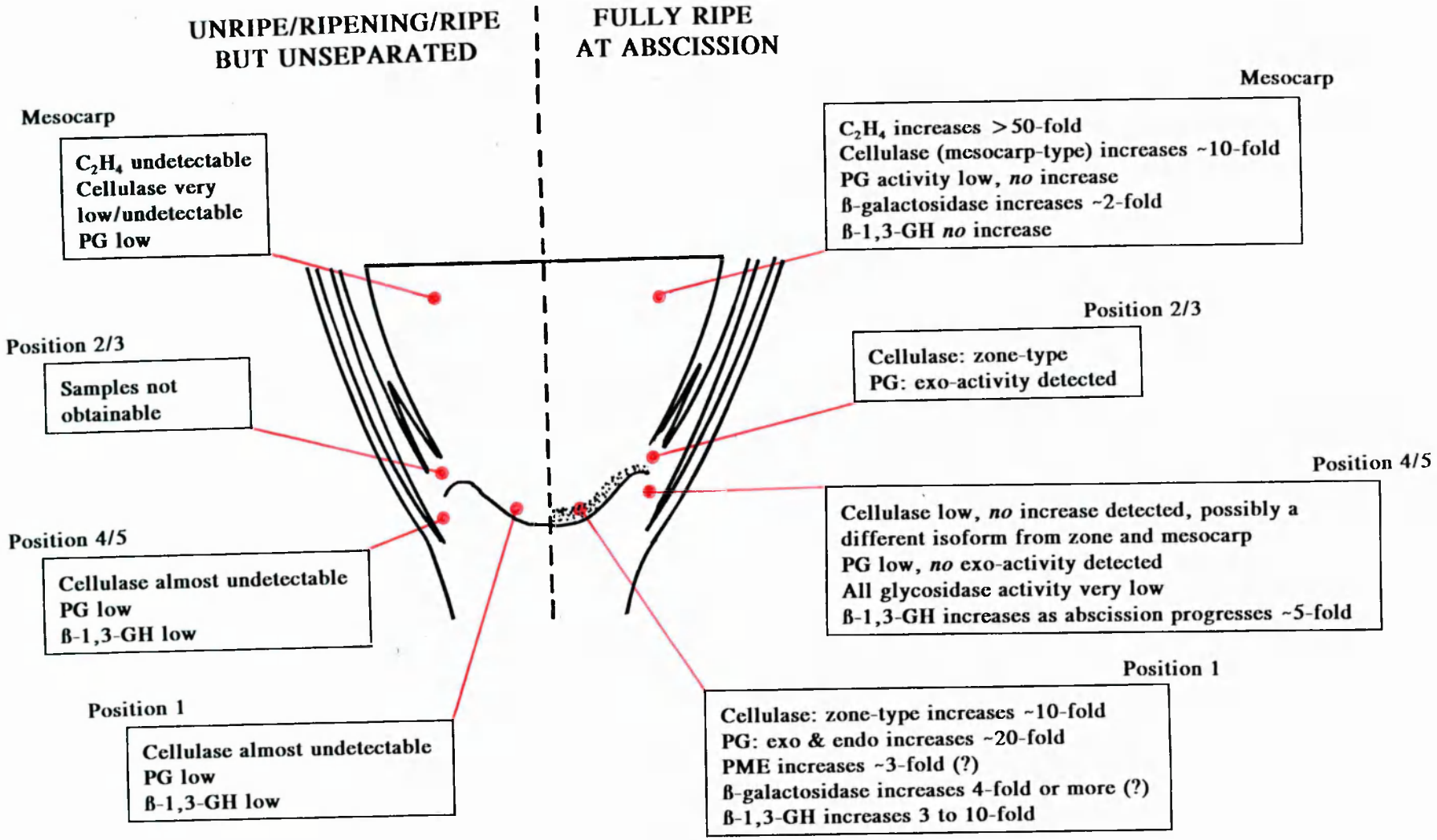


FIG. 10.1: SUMMARY OF THE ENZYME ACTIVITIES BEFORE AND AFTER ABSCISSION IN THE OIL PALM FRUIT

10.1.1 A summary of what is now known about ripening and abscission in oil palm fruit

Ethylene is not detected in fruit during the ripening period. This was established with both individual fruit and whole spikelets. When the spikelets arrived in the laboratory they had been excised from the palm for at least 24h and yet, for the majority of **unripe and ripening** fruits, there was no measurable ethylene production. This indicates that the carotenoid, triacylglycerol and lipase biosynthesis during the ripening period is probably not under the control of ethylene. However, at a critical stage of **full ripeness** high levels of ethylene are produced which in turn signals the initiation of abscission. Whilst an active cellulase is induced in the mesocarp tissue concomitant with high ethylene production, no induction or increased activity of polygalacturonase occurs in the mesocarp. The first sign of imminent cell separation at the abscission zone is that the pectin/polygalacturonate-rich zone becomes translucent. This is possibly due to pectin methylesterase activity (since treatment of unseparated fruit with EDTA, an inhibitor of polygalacturonase activity, does not prevent the zone from becoming translucent). A zone-specific cellulase (different from the cellulase induced in the mesocarp) together with both exo- and endo-polygalacturonase isoenzymes (two exo pI 6.2-6.4 and two endo pI 9.0, 9.3 isoforms) are induced along with an increased ß-1,3-glucanase and ß-galactosidase activity. Within several hours these activities culminate in cell separation at the zone (Positions 1+2/3). This is then followed some 24h later in the separation of tepal bases from the pedicel at Position 4/5. The relative enzyme activities detected in tepal abscission (4/5) are very different from those expressed in Position 1+2/3 zone abscission. In Position 4/5, cellulase, endo-polygalacturonase (an absence of exo-polygalacturonase activity) and all the exo-glycosidases tested have very low activities. Only ß-1,3-glucanase reaches high levels in Position 4/5. In addition, the cellulase isoenzyme(s) induced in Position 4/5 appear(s) to have characteristics different from both the mesocarp and zone-type cellulases and may be the product of a separate tissue-specific gene expression.

The differences in enzyme activities between normal unripe/ripening/ripe **unseparated** fruit and fully ripe fruit **undergoing natural abscission** are illustrated in Fig. 10.1.

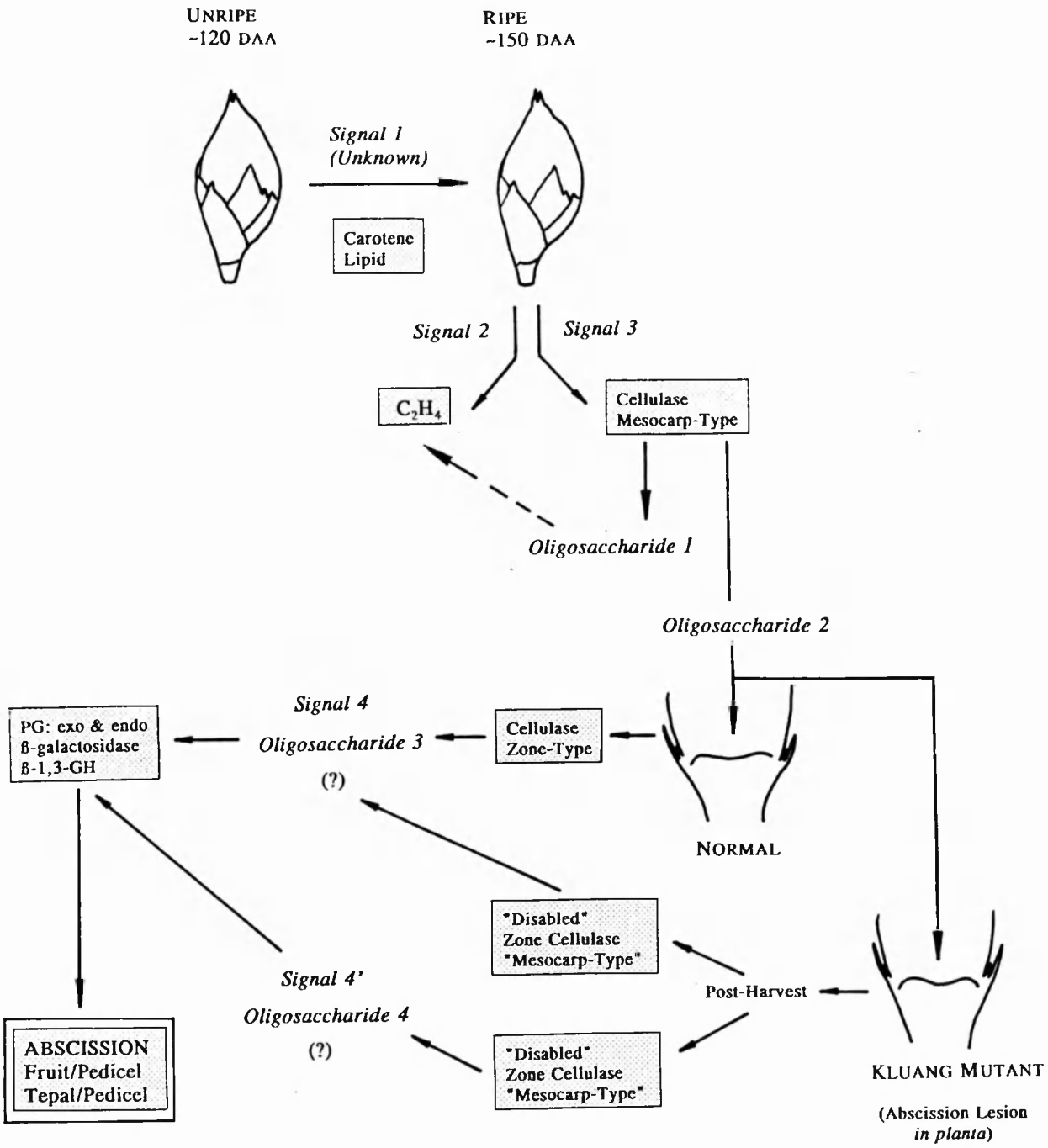


FIG. 10.2: A SIGNALLING MODEL IN NORMAL AND MUTANT (NON-SHEDDING) FRUIT ABSCISSION

The discovery of non-abscinding mutants has made it possible to determine which enzyme activities were different when compared with normal fruit and those now open to gene manipulation to improve the shedding regime of the plantation palm. The zone-specific cellulase was shown to be altered in the mutant and possibly "disabled" enzymically on its zone substrate or another cellulase isoform is induced as a post-harvest event ("late" cellulase). Also, pectin methylesterase activity in all the mutant tissues was lower than in the normal, whereas ß-1,3-glucanase activity was much higher. Although the mutant fruit did eventually undergo a slow separation, it appeared that these enzymic differences resulted in an altered pectin disassembly in the cell walls of the abscission zone.

10.1.2 A model of abscission in the oil palm (Fig. 10.2)

An unknown signal initiates ripening in the oil palm fruit whereby biosynthesis of TAG, carotene and lipase occurs. The fully ripe mesocarp is the site of ethylene production and the induction of cellulase. Does ethylene induce cellulase or does cellulase produce a signal which results in the induction of ethylene? Application of the ethylene inhibitor, aminooxyacetic acid, to unripe and ripening fruit reduces the post-harvest increase in ethylene production and then zone separation does not occur (Henderson & Osborne, 1994), suggesting that ethylene is indeed the trigger for abscission. But a question remains: What is the signal that induces the rise in ethylene biosynthesis?

At full ripeness, ethylene and other signals passed from the mesocarp (possibly via the phloem) is/are perceived at the abscission zone. This results in the induction of a zone-specific cellulase. In the bean, the pI 9.5 abscission cellulase is first induced in the stele and subsequently in cortical cells as abscission progresses. Removal of the stele prevents the induction of the pI 9.5 cellulase in cortical cells and stops abscission. Thus, in bean the action of the pI 9.5 cellulase itself may produce an abscission-promoting oligosaccharide. Since in oil palm the vascular bundles are scattered throughout the abscission zone, any abscission-promoting oligosaccharide produced by the zone cellulase will be relatively evenly distributed and may well signal, along with ethylene, the induction of high activity in polygalacturonases. A concerted action of both exo- and endo-hydrolysis together with the other induced enzymes would no doubt result in rapid and complete dissolution of the middle lamella pectin and expansion by wall loosening of

the subtending neighbour cells.

Abscission of fruit on the Kluang mutant palm on the plantation does not occur. Both ethylene and mesocarp-type cellulase are produced in the ripe mesocarp tissue as in normal fruit. These mutant fruit are able to separate only after removal from the parent palm. The cellulase induced in the zone of the post-harvest fruit resembles the mesocarp-type in its pH profile and not the normal zone cellulase, which may suggest that it is "disabled" in some way and takes longer or requires different conditions in the apoplast in order to hydrolyse the mutant middle lamella and wall components. After harvesting, the loss of water by the transpiring fruit must cause some perturbation in the system; increased callose synthesis is, therefore, possible and the then very high level of ß-1,3-glucanhydrolase activity combined with a post-harvest change in the osmotic environment in the apoplast (the cell wall may swell or shrink), makes it possible for the "disabled" cellulase to function either as a wall loosener by wall hydrolysis or to produce an oligosaccharide signal molecule.

10.2 FUTURE PROSPECTS AND FURTHER RESEARCH

Manipulation of shedding has always been a desirable goal in oil palm plantation management. If the abscission of outer fruit (which ripen first) is prevented or retarded, the inner and central fruit would then reach full ripeness before the whole bunch is harvested. In addition, increased costs are incurred to pay harvesters to collect the individual shed fruit around the palms. The non-abscinding Kluang mutant and others (like Sabah Palm 2) are currently being used in breeding trials to achieve this aim. It remains to be shown whether the progeny of these mutants will improve bunch harvesting and harvested oil yields.

Both fruit abscission and harvesting the bunch adversely affect the quality of the product obtained from the fruit (that is, the mesocarp triacylglycerols) so that they cannot be stored (unlike many other fruit). The oil must therefore be extracted as soon as possible after harvesting. The focus of research on the oil palm to date has been to improve quantitative yields by plantation management and to improve the quality of palm oil with investigations relating to the type and composition of triacylglycerols of the ripe

fruit. Future research may well focus on the genetic manipulation of developmental processes in the oil palm; specifically those of ripening and shedding. A good biochemical understanding especially of tissue-specific enzyme activities is essential before any new gene functions and their regulation can be undertaken. Whilst such information has been available for ripening in the tomato, it is only in recent years that the biochemistry of ripening and shedding of oil palm fruit has been seriously researched.

Methods for genetic transformation with the introduction of foreign genes into oil palm is now beginning (Chowdhury *et al*, 1997). Antisense RNA to inhibit specific gene expression *in planta* may prove to be a valuable tool for its genetic improvement. For example, an important point of control in oil palm is immediately before abscission starts in the fully ripe fruit, when both the production of ethylene and the induction of mesocarp cellulase link temporally with the hydrolysis of the valuable triacylglycerols at abscission. Antisense ACC oxidase or synthase, with subsequent down regulation of ethylene synthesis, might well retard abscission (especially of outer fruits) and achieve synchronous ripening along with continued oil synthesis. Whilst the function of the mesocarp lipase in lipid synthesis during ripening has not been fully investigated, its hydrolytic activity at abscission is clear. If it is not directly involved in lipid production, then antisense technology applied to this enzyme, too, would reduce free fatty acid formation after abscission with higher triacylglycerol yields and no spoilage after harvest.

A further potential improvement relates to the palm fronds. These need to be cut from the palm as it grows, so that fruit bunches can be observed from the ground and harvested at a properly ripe stage. Palms like the Royal Palm have a functional abscission zone at the leaf base, and the large fronds abscind leaving a smooth trunk and a clear view of the inflorescences above. The identification of promoters that will regulate gene expression for abscission cellulases and polygalacturonases in the specific cells at the base of the frond in the oil palm, so introducing a positionally differentiated and operative abscission zone, would have enormous economic potential.

The information in this thesis hopefully provides a basis to achieve some of these developmental possibilities for improving the oil palm as an even more successful plantation crop.

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APPENDIX A

SOLID STATE ^{13}C CP-MAS NMR ANALYSES
by Dr S.J. Heyes, Inorganic Chemistry Department, Oxford University

^{13}C CP-MAS spectra were obtained on a Bruker MSL 200 NMR spectrometer at Larmor frequency for ^{13}C of 50.32 MHz. Chemical shifts are referenced to an external secondary standard (adamantane upfield resonance = 29.5 ppm). The dried, ground samples (200-300mg) were placed in 7mm zirconia rotors with Kel-F caps. The 90° pulse length = 4μs. Spectra were accumulated with up to ~3,000-6,000 transients with a 4s recycle delay between transients. MAS rates of 3 to 5 kHz were used, and the 5 kHz rate is presented. NQS, non-quaternary suppression, (dipolar-dephasing) experiments were performed with 50μs delay for dephasing (with 180° pulse for refocusing CSA effects in the middle). ^{13}C CP-MAS NMR spectra were performed on the 124 daa zone scrapate sample with a range of cross polarisation contact times from 0.5 to 10ms. An optimum time of 2ms was chosen as offering the best compromise between the short (0.75ms) optimum contact time of the rigid carbohydrate resonances and the long (5-10ms) values for mobile and non-protonated carbons, and was used for all subsequent spectra. FIDs were zero-filled to 16k points prior to Fourier transformation, and no apodization functions were used.

References for these techniques are found in Edwards *et al* (1995).

- **The Mesocarp Tissue (Fig. I)**

Unripe (61 daa), ripening (124 daa) and ripe mesocarp (152 daa) tissues are compared in Fig. IA-C. In the spectrum of the unripe mesocarp (A) the resonances for the 'carbohydrate' of cell wall material between 60-110 ppm and resonances between 170-180 ppm are similar to those of ripening tissues (mesocarp directly above the zone, unseparated zone and pedicel) shown in Fig. IIA-C. However, in contrast to the mesocarp directly above the zone in ripening fruit (Fig. IIA), unripe mesocarp has no distinct peak at ~54 ppm for the methoxyl group in pectin and/or lignin (NQS spectrum, Fig. IA). Since the resonances for lignin are absent in unripe mesocarp, this suggests that the pectin is largely unmethylesterified (below the limit of detection by ^{13}C NMR). This also is supported by more intense staining of polygalacturonate by Ni^{2+}/Na_2S in the mesocarp of

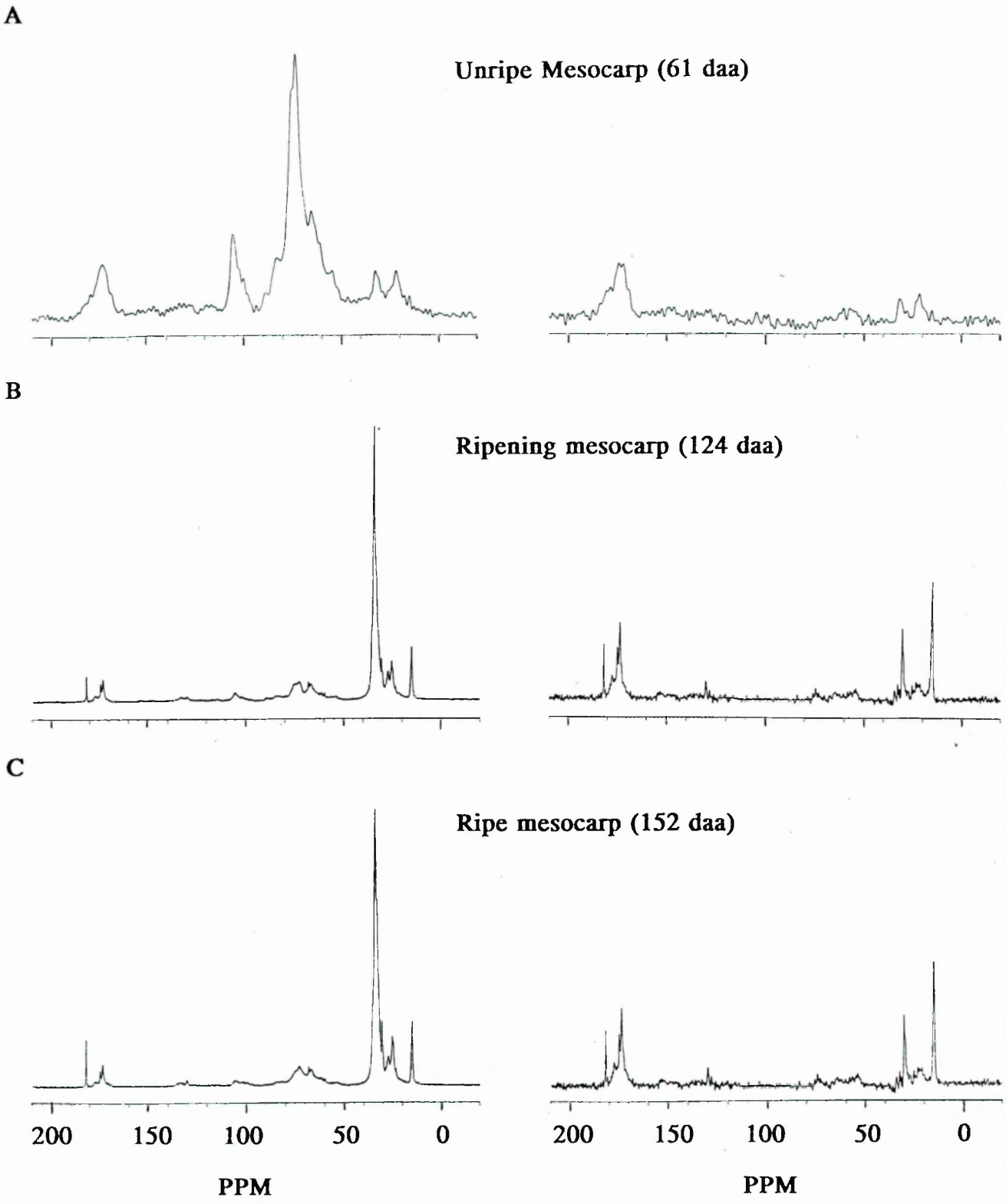

FIG. I: ^{13}C CP-MAS NMR SPECTRA OF UNRIPE, RIPENING AND RIPE MESOCARP (NORMAL FRUIT.

The spectrum on the left is of all ^{13}C resonances and on the right, an NQS-Delay spectrum. T resonances from lipid (15-40 ppm; 170-185 ppm) predominate in the spectra of ripening (B) and r (C) mesocarp and those from the cell wall material (60-100 ppm) are much less intense relative to major lipid component. In contrast, in the unripe mesocarp (A) resonances from cell wall material (100 ppm) show this is the major component with less intense resonances (15-40 ppm) from a very l amount of lipid.

young unripe fruit compared with that in ripening fruit (Section 3.5.1, Fig. 3.12C and D, page 91A).

Ripening mesocarp (124 daa) is only just beginning to synthesize fatty acids and deposit triacylglyceride (compare Fig. 1.5C, page 32A). However, the concentration of fatty acids/TAG even at this early stage is already high compared with the cell wall material. Figure IB shows that the intensity of the resonances between 60-110 ppm, mainly from cell wall carbohydrate, is very low in contrast with those between 15-40 ppm, from methylene and methyl groups in fatty acids. The peaks between 172-182 ppm may be attributed to the three ester groups in triacylglyceride and the free carboxyl in fatty acids. Fully ripe mesocarp from separated fruit (152 daa which is rich in triacylglyceride and fatty acids, Fig. 1.5C, page 32A), has essentially the same spectrum as ripening mesocarp substantiating these identity of these assignments (compare Figs. IB & C).

These spectra of unripe, ripening and ripe mesocarp confirm the presence of fatty acid/TAG as fruit begin to ripen (~120-130 daa). They show also that resonances from the major components of a tissue can be distinguished in the spectrum, and although lipid deposition is only just beginning at 124 daa, its presence is readily detectable with resonances of much higher intensity than those of cell wall material.

- **Comparison of Mesocarp, Pedicel and Unseparated Zone Tissues (Fig. II)**

Figure II compares the normal ripening unseparated zone (B) with the tissues directly above and below it (mesocarp (A) and pedicel (C)). The spectrum of unseparated zone (Fig. IIB) includes contaminating mesocarp and pedicel tissues, which together probably comprise about two-thirds of the zone sample. When compared with the mesocarp tissue adjacent to it (Fig. IIA), the group of resonances of unseparated zone at 15-40 ppm is poorly represented. Both pedicel and mesocarp components of an unseparated zone contain many more lignified fibre elements than the zone itself (Section 3.3). It is not surprising, therefore, that aromatic resonances (115-165 ppm), particularly those between 120-140 ppm, from lignin are more intense in both pedicel and mesocarp than the zone (compare the NQS-delay spectrum, Fig. IIB and C). These differences confirm that the unseparated zone sections, although containing tissues other than the zone, do present a ^{13}C NMR spectrum that is distinguishable from the adjoining mesocarp and pedicel.

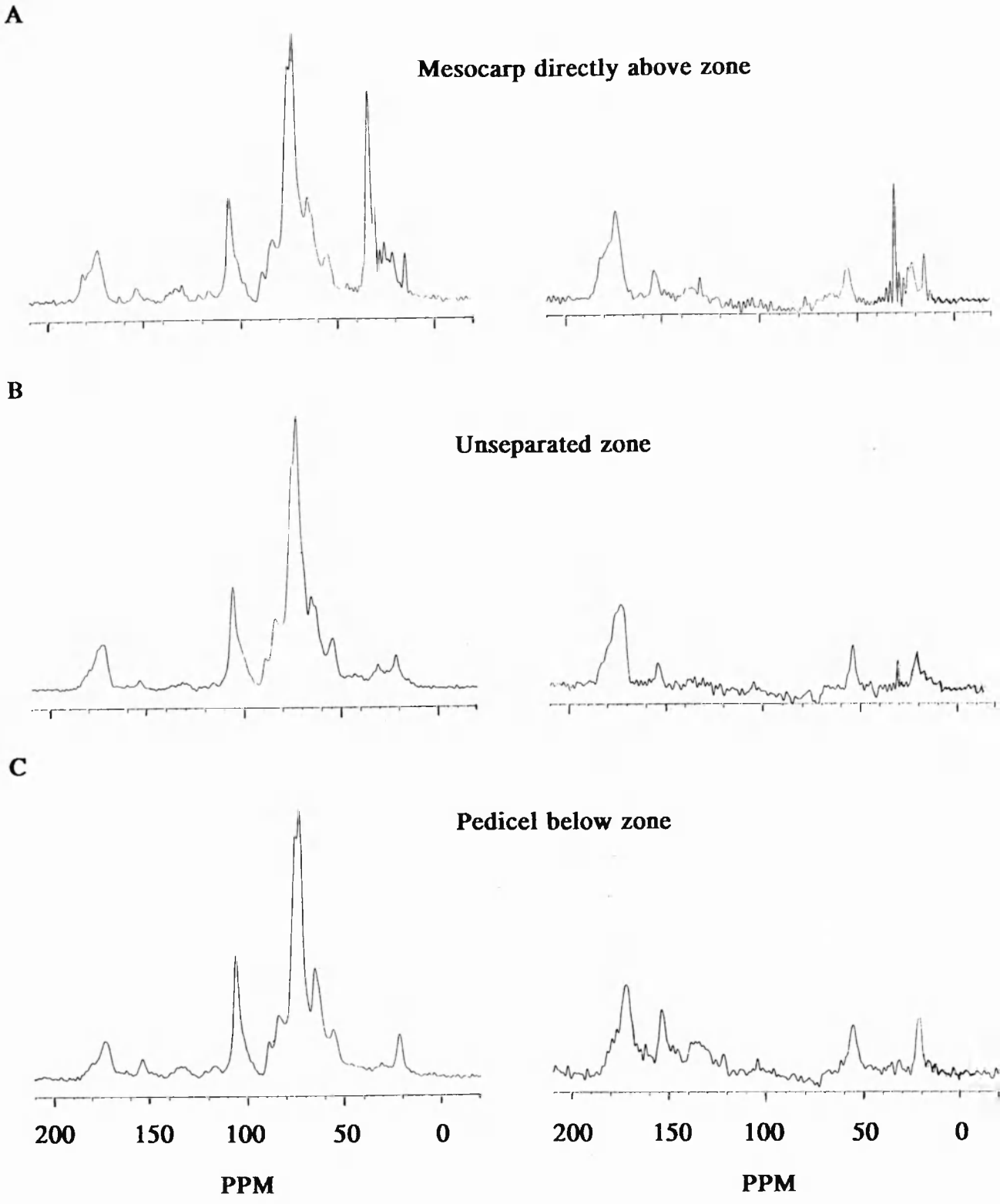

FIG. II: ^{13}C CP-MAS NMR SPECTRA OF MESOCARP DIRECTLY ABOVE THE ZO UNSEPARATED ZONE AND PEDICEL TISSUE BELOW THE ZONE.

The spectrum on the left is of all ^{13}C resonances and on the right, an NQS-Delay spectrum. mesocarp directly above the zone (A) contains some lipid (15-40 ppm) and these resonances are intense in the unseparated zone tissue, indicating that there is only a small amount of mesocarp pr in the unseparated zone slice. Resonances from lignin (115-165 ppm) are present in pedicel tissue all three tissues contain a similar amount of methoxylated ^{13}C (~54 ppm).

It is interesting to note that the mesocarp directly above the zone has a very different spectrum from mesocarp taken from the central region of the same fruits (compare Fig. IIA with IB). Although in the mesocarp directly above the zone, the intensity of the group of resonances between 15-40 ppm (methylene and methyl groups in fatty acids/TAG) is similar to that in mesocarp from the central region (Fig. IB), resonances for the cell wall material between 60-110 ppm are markedly greater in the mesocarp directly above the zone (Fig. IIA). Therefore, the intensity of these resonances relative to each other indicates that there is a greater concentration of lipid in the mesocarp from the central region compared with that in mesocarp directly above the zone.

The peak between 50-60 ppm (~54 ppm) is generally assigned to the ^{13}C in methoxyl groups. These are present in methylesterified galacturonan and in lignin (syringyl, guaiacyl, coniferyl and sinapyl constituents all contain methoxyl groups). In the mesocarp, unseparated zone and pedicel tissues (Fig. IIA-C), it is not known whether the contribution is greater from the methylesterified galacturonan or from the lignin. However, in the spectrum of normal separated zone (Fig. 3.15A, page 95A), there is an absence of aromatic resonances (115-165 ppm) which allows the small peak at ~54 ppm to be assigned to the methoxyl group in methylesterified pectin. This also is confirmed by the low level of phloroglucinol/HCl staining of lignin in the few attenuated xylem vessels which pass through the zone (and contribute to $<8\%$ its area, Fig. 3.10, page 88A; Section 3.3).

The broad peak between 170-180 ppm (Fig. IA-C), in contrast to the galacturonan standards (Fig. 3.14, page 94A) does not clearly differentiate the methylesterified carboxyl group in pectin (~171 ppm) from the free carboxyl in polygalacturonate (~177 ppm). Resonances from other esters, carboxylic acids or amides may also contribute but the chemical analysis of uronic acid and Ni^{2+}/Na_2S staining of polygalacturonate in mesocarp, zone and pedicel suggests that the major signal is from the carboxyl group in pectin. The intensity of the 170-180 ppm peak relative to the other peaks in the spectrum indicates that pectin is **not** a minor component in the cell walls of oil palm fruit tissues, as has been shown for some monocotyledonous plants (Jarvis *et al*, 1988).

APPENDIX B

FIXATION OF MATERIAL FOR LIGHT AND ELECTRON MICROSCOPY

by Mrs H. Davies, Electron Microscopy Unit, Open University, Milton Keynes.

B1. Freeze substitution (Figs. 3.6-3.8)

1. Hand-cut sections were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M Sorensons phosphate buffer, pH 7.3 overnight on a rotator at room temperature.
2. Sections cryo-protected by increasing concentrations of glycerol in phosphate buffer: 10%, 20% and 30% for 1h each.
3. Sections were impact frozen onto a polished copper mirror and freeze-substituted in methanol at -85°C for 65h and at -50°C for 22h in a Leica AFS freeze substitution apparatus.
4. Sections were infiltrated with Lowicryl HM20 at -50°C; 1:2, 1:1 and 2:1 HM20: methanol for 1h each.
5. Sections were subjected to three changes of Lowicryl HM20 (one of which was overnight) before embedding in flat-bottomed BEEM capsules and polymerising under ultraviolet light for 48h at -50°C.
6. 70nm ultrathin sections and 1μm semi-thin sections were cut on a Leica OMUE Ultramicrotome.
7. The semi-thin sections were dried onto a microscope slide and stained with toluidine blue (1% toluidine blue in 5% borax) for approx 10secs at 70°C.
8. The ultrathin sections were stained with uranyl acetate and lead citrate in a LKB Ultrastainer.

B2. Potassium Permanganate Fixation (Fig. 3.5)

1. Hand-cut sections were fixed in 1% aqueous potassium permanganate for 2h at room temperature, washed several times in distilled water and left overnight in 0.1M phosphate buffer.
2. Sections were dehydrated through a graded series of ethanol: 10%, 20%, 30%, 50%, 70%, 90% and two changes of 100% for 30min each.
3. Soak sections in propylene oxide for 2x15min.
4. Infiltrate with increasing concentrations of Epon resin:propylene oxide; 15%, 30%, 50%, 75% for 15min each.
5. Epon overnight.
6. Epon for 6h. Embed in BEEM capsules and polymerize at to 60°C for 24h.

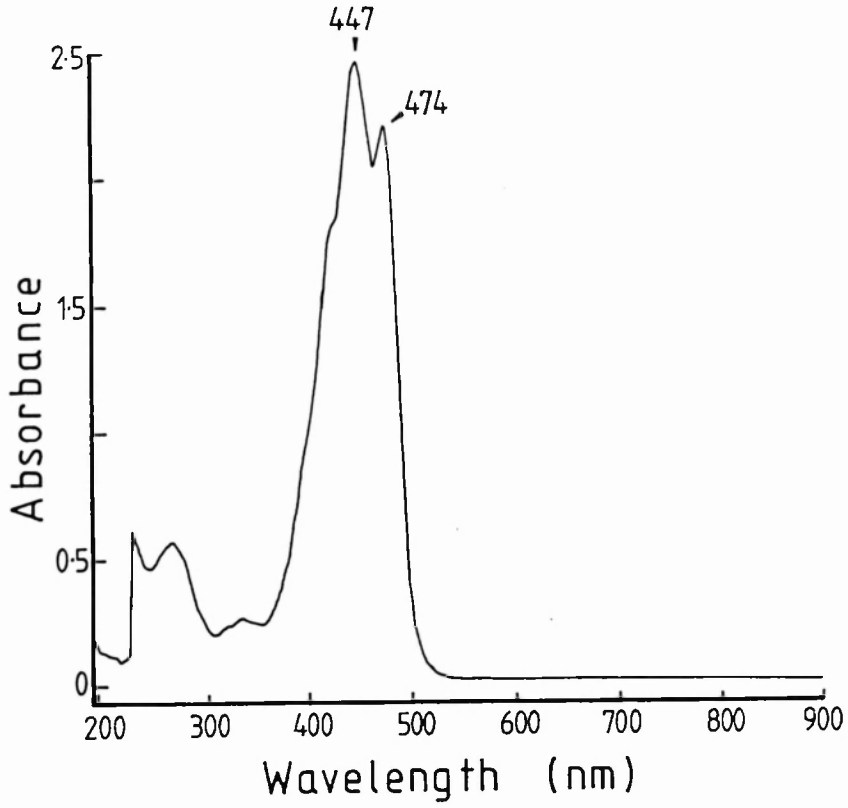

FIG. III: WAVELENGTH SCAN (200-900NM) OF CAROTENOIDS EXTRACTED (AS DESCRIBED IN APPENDIX C) FROM RIPE MESOCARP

APPENDIX C

EXTRACTION AND ANALYSIS OF CAROTENOIDS

The carotenoid content in unripe, ripening and ripe mesocarp was extracted by the method used for oil palm described by Ikemefuna & Adamson (1984) and references therein.

For each analysis, mesocarp slices (1g) were cut from the mid-region of 3-4 fruit. The chopped slices were ground with liquid nitrogen to a fine powder using a cooled mortar and pestle. The pigments were removed by extracting with 100% acetone (2-3ml) and filtering through a scintered glass funnel. The debris was re-extracted 3-4 times until all colour had been removed. The extract was placed in a separating funnel and an equal volume of diethylether was added. Deionised water was then added until two layers formed. The upper ether layer was retained and treated with 60% methanolic KOH (w/v); 2ml methanolic KOH was added for each 10ml of acetone extract. This saponification step removes the lipid (TAG). After standing 20-30min, an equal volume of 1% NaCl was added to the extract and then the lower layer released and discarded. Chlorophyll contamination of the carotenoids in the ether layer was removed by treatment with 4% NaCl. The ether layer was then retained and dried over anhydrous Na_2SO_4.

A wavelength scan of a ripe mesocarp extract prepared by this method shows that the major absorption peaks are at 447nm and 474nm corresponding to the literature λ_{max} for carotene (α and ß). ß-carotene has two visible wavelength absorption maxima in petroleum ether: 453nm (ϵ = 130,000) and 483nm (ϵ = 111,000). α-carotene has an absorption maxima at 444nm.

The absorbance of the total carotenoid content extracted was determined at 440nm (Cecil CE 4400 UV/Vis double beam spectrophotometer) and a quantitative estimate of the total carotenoids (μg ml^{-1}) was obtained by dividing the absorbance by 0.24 (see Boardman & Anderson, 1967, in Ikemefuma & Adamson, 1984). This was confirmed using the molar absorbivity co-efficient for ß-carotene (ϵ = 130,000) and total carotene in μg g Fw^{-1} was calculated.

APPENDIX D

COMMENTS ON FRUIT SHEDDING IN THE NON-ABSCINDING PALMS BY PAMOL PLANTATIONS, MALAYSIA.

PAMOL HQ 07 779120 08-02-96 07:01 [31 #1

PAMOL PLANTATIONS SDN. BHD.
(DITUBUHKAN DALAM MALAYSIA)

Tel 07-7879311 (10 Lines)
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P. O. BOX 1,
86007 KLUANG,
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CRD/ocs

7 Feb 1996

Dr D.J. Osborne
Oxford Research Unit
The Open University
Foxcombe Hall, Berkeley Road
Boars Hill, OXFORD, OX1 5HR
U.K.

Fax : 007-44-1865-326 322
(One page only)

Dear Dr Osborne

Chew will also ask our field staff. Personally I have not seen any GOOD fallen fruit on any of the four palms.

Best wishes.

(CHRIS DONOUGH)

REGISTERED OFFICE: PUSAT PAMOL, UNGANG PAMOL SATU, 81, JALAN MERSING, 86000 KLUANG, JOHOR, MALAYSIA.

PAMOL HQ 07 779120 27-02-96 08:43 [25] #1

PAMOL PLANTATIONS SDN. BHD.
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Our Ref : CKW/ocs

Date : 26 Feb 1996

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Fax : 007-44-1865-326 322
(~~Four~~ One pages only)

Dear Dr Osborne,

I refer to your fax dated 23 February 1996. Rengasamy will keep an eye on fallen good fruit from the non-abscinding palm in Kluang. According to him, the few good fruits that shed in the field came from the center and bottom of the spikelets.

Bunches 19 and 20 were around 75% rotten and therefore harvested. The detached fruits on the ground were rotten. For bunch 21, a few days before it was harvested, Rengasamy (our field staff) saw a few rotten and GOOD fruits on the ground. The bunch was not rotten. There was no detached fruit for bunch 22 and the bunch was not rotten.

In Kluang, most of the fruits dropped were rotten and cracked. However, Rengasamy had observed that a few non-rotting/GOOD fruits dropped to the ground. Nevertheless, these fruits were cracked. For Sabah, our staff had noticed that a few of the fallen fruits were GOOD and the others were rotten from Palm 1. For Palm 2, all loose fruits on the ground were rotten.

(CHEW KOK WAI)
Research Manager

APPENDIX E

PREVIOUS PUBLISHED WORK ON THE ANATOMY AND BIOCHEMISTRY OF THE OIL PALM FRUIT

Spruce, J., Mayer, A.M. & Osborne, D.J. (1987) A simple histochemical method for locating enzymes in plant tissue using nitrocellulose blotting. *Phytochemistry,* **26** : 2901-2903.

Henderson, J. & Osborne, D.J. (1990) Cell separation and anatomy of abscission in the oil palm, *Elaeis guineensis*, Jacq. *J. Exp. Bot.* **41** : 203-210.

Henderson, J. & Osborne, D.J. (1991) Lipase activity in ripening and mature fruit of the oil palm. Stability *in vivo* and *in vitro*. *Phytochemistry*, **30** : 1073-1078.

Osborne, D.J., Henderson, J. & Corley, R.H.V. (1992) Controlling fruit shedding in the oil palm. *Endeavour, New Series*, **16** : 173-177.

Henderson, J. & Osborne, D.J. (1994) Inter-tissue signalling during the two-phase abscission in oil palm fruit. *J. Exp. Bot.* **45** : 943-951.

Phytochemistry, Vol. 26, No. 11, pp. 2901–2903, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

A SIMPLE HISTOCHEMICAL METHOD FOR LOCATING ENZYMES IN PLANT TISSUE USING NITROCELLULOSE BLOTTING

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(*Received* 30 *March* 1987)

Key Word Index—Nitrocellulose blotting; histochemistry; plant enzymes; abscission zones.

Abstract—The blotting of fresh tissue sections onto nitrocellulose and the detection of enzyme activities on the blots for polyphenol oxidase, peroxidase, glycosidases, dehydrogenase and phosphatase activity has been shown successfully using the method. The applicability to various tissues and enzyme localization is discussed.

INTRODUCTION

The histochemical and phytochemical localisation of plant enzymes is well developed at the microscopic level [1] but can nevertheless often be time consuming and tedious. The plant tissues frequently contain the native substrates of the enzymes to be detected or compounds that specifically inhibit the enzymes. In addition many plant tissues contain substances, such as phenolics, which bind to the proteins and generally inhibit enzyme activity. The speedy localization of enzyme activities in tissue sections is often desirable as well as a relative measure of the activity between different cell types as, for example, in abscission zones [2].

A simple rapid method to achieve this without first processing the tissue, and without interference by inhibitors or substrates would therefore be very valuable. We describe such a method utilising the properties of nitrocellulose to absorb and retain proteins, as used in various molecular biological blotting techniques.

RESULTS AND DISCUSSION

As can be seen from Fig. 1 sections through the petiole of the primary leaf of the bean (*Phaseolus vulgaris*) in the region of the distal abscission layer or of the lower part of oil palm fruit (*Elaeis guineensis*), also in the region of the abscission zone, clearly show that it is possible to detect enzyme activities blotted on to nitrocellulose. The blotting was achieved by simply pressing a cut section lightly on to the membrane and then developing with specific histochemical reagents. The data in Fig. 1 show representative examples of the assay for some of nine enzymes and in the two different tissues.

We have assayed for α- and β-glycosidase, α- and β-galactosidase, acid phosphatase, polyphenol oxidase, peroxidase, and glutamate and malate dehydrogenase. The localisation of some of the enzymes and the wide spread of others, can be seen.

For example, polyphenol oxidase (Fig. 1a) and phosphatase (not shown) are present throughout the oil palm section while β-glucosidase is detected only in the abscission zone (Fig. 1b). Although some peroxidase is present throughout the section, a clear zone of high activity was noted in the abscission zone (Fig. 1c). In the bean petiole polyphenol oxidase (not shown) and glutamete dehydrogenase activity were observed particularly in the region of the pulvinus (Fig. 1f) while peroxidase (Fig. 1d) is widely distributed. Differences in activity could be clearly seen on either side of the abscission layer.

The method should be applicable for any enzyme which can be transferred to nitrocellulose and for which a histochemical assay exists, using a substrate that results in the formation of insoluble or partially soluble coloured or fluorescent product.

Microscopical examination of the tissue blots indicates that proteins located in individual cells are giving enzyme reactions. Indeed different cell types such as vascular tissue can be readily distinguished on the blots; the restriction of β-glucosidase to the abscission layer of the oil palm is an example.

The method should find wide application for the localisation of enzymes in specific differentiating or differentiated plant tissues and could be particularly useful in phytopathological studies where it is desired to locate host or parasite enzyme in tissues.

EXPERIMENTAL

2 cm squares of nitrocellulose membrane filters B A 28, (Schleicher & Schüll, 0.45 μm pore size) were soaked in distilled H_2O, placed on microscope slides and blotted dry with a tissue. A 2–3 mm section through the plant tissue was placed on the membrane and then lightly pressed on it using an additional microscope slide. The section was removed carefully and the membrane thoroughly rinsed with distilled H_2O to remove non-proteinaceous material or soluble compounds and lightly pressed with a tissue to remove excess water. About 0.2 ml of the appropriate substrate or reagent was next spread over the surface of the membrane. The slides were incubated in a moist atmosphere for the reaction to proceed, the substrate was then rinsed off, and if necessary a developing reagent added. For fast reactions with immediate colour production the substrate was

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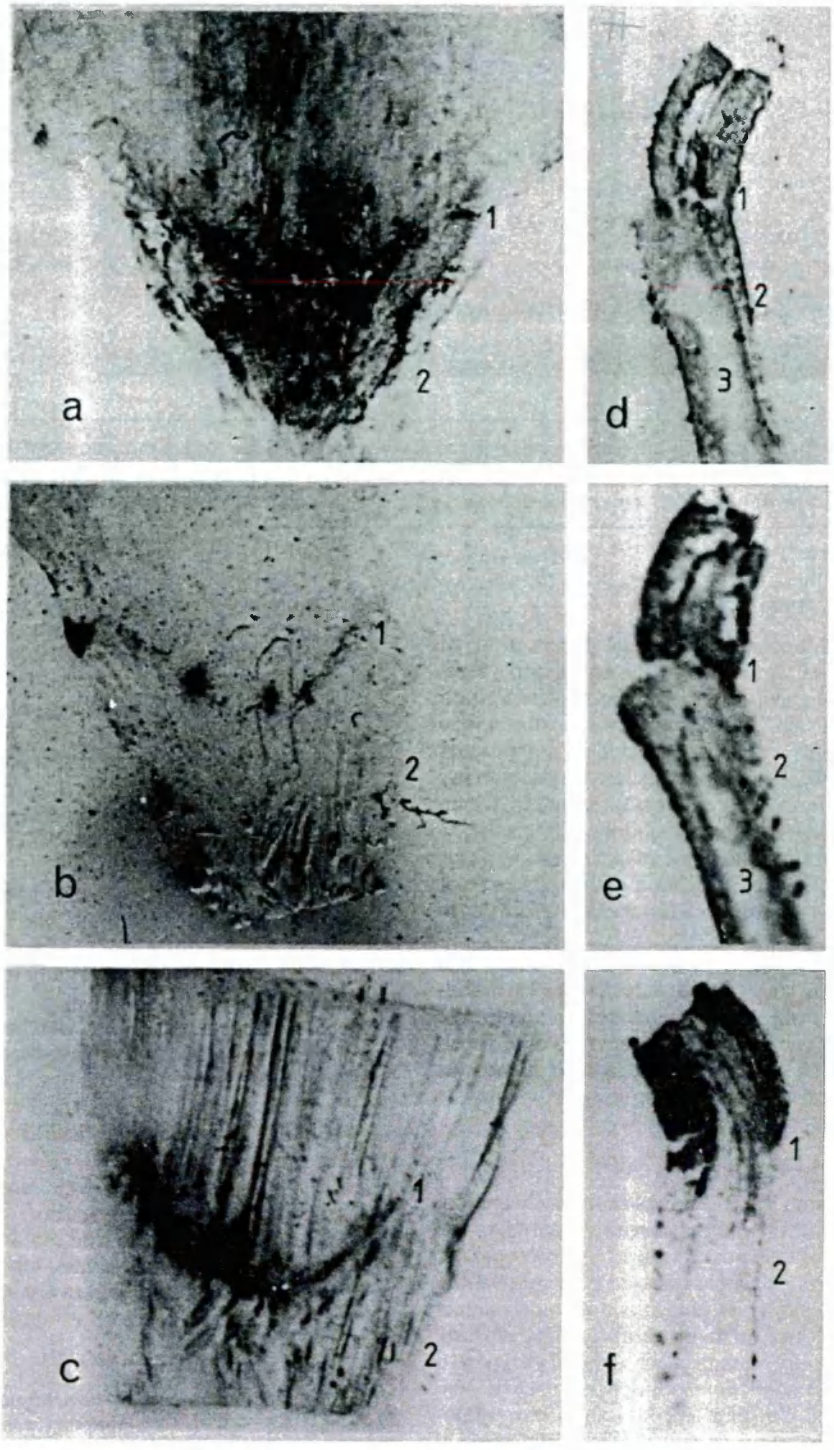

Fig. 1. Photographs of histochemical blots of oil palm fruit and bean leaf petioles. (a–c) Sections through oil palm fruit across fruit across abscission zone. (d–f) Sections through petiole, abscission zone and pulvinus of bean. (d, e, partially separated zones). (a) Polyphenol oxidase; (b) β-glucosidase; (c, d) peroxidase; (e) malate dehydrogenase; (f) glutamate dehydrogenase. Darkened or black areas show sites of enzyme activity. 1 = Abscission zone; 2 = proximal side; 3 = hollow pith (bean petiole).

added and rinsed off as soon as colour development was optimal, e.g. polyphenoloxidase or peroxidase. Alternatively the nitrocellulose blot can be placed (blot side upwards) on to a piece of filter paper moistened with substrate. This gives very good resolution and localization of enzyme activity.

For work with oil palm fruit the substrates were made up in pH 4.8 acetate buffer 20 mM, and for the bean leaf petioles pH 6.1 Pi buffer, 100 mM. We have used the following reagents from Sigma, London for detecting enzyme activity: for polyphenol oxidase the reagent was 3,4-dihydroxyphenylalanine 20 mM [3], for per-

oxidase we used 0.2% guaiacol and 0.2% H_2O_2, but 3,3'-diaminobenzidine is equally effective. For α- and β- glucosidase and galactosidase the substrates were the corresponding bromo-naphthol glycosides as described in refs [4, 5] using 3 mg/10 ml. For acid phosphatase we used 1 mg/2 ml naphthol-AS-BI-phosphate. In these tests the substrate was washed off after incubation for some 3–4 hr at 26° and the visualizing reagent Fast Blue B 1 mg/ml added and left on the membrane for 4 min. The dehydrogenases were detected with tetrazolium nitroblue 0.03% in the presence of 0.002% PMS, 1.0 mM NAD and 150 mM glutamate or 100 mM malate [6]. All membranes were finally rinsed, excess H_2O removed with a tissue, and air-dried. Placed between two slides membranes could be kept in the dark for several weeks without deterioration.

The dried membranes are readily photographed, using appropriate light filters.

Acknowledgement—We wish to acknowledge financial support from Unilever Plantations Group.

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6. Shaw, C. R. and Prasad, R. (1970) *Biochem. Genet.* **4**, 297.

Note added in proof: With C. C. McCready we have now successfully extended this tissue blot method for cell recognition by immunogold blotting procedures using Janssen Auro Probe BL Plus followed by silver enhancement.

Journal of Experimental Botany, Vol. 41, No. 223, pp. 203–210, February 1990

Cell Separation and Anatomy of Abscission in the Oil Palm, *Elaeis guineensis* Jacq.

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Received 13 July 1989

ABSTRACT

Shedding of the fruit of the oil palm takes place in two co-ordinated stages. The first, a cell separation event at a pre-defined, positionally differentiated abscission zone at the base of the fruit, is followed by further cell separation in peripheral tissue at the junction with the rudimentary androecial ring and the tepals. The position of the second separation is determined by the age and ripeness of the fruit and the degree of pressure to which it is subjected; it is also dependent upon completion of the first stage. Implications of this unusual two stage separation process are discussed.

Key words: Abscission, cell separation, anatomy, oil palm, *Elaeis guineensis*.

INTRODUCTION

The oil palm, *Elaeis guineensis* Jacq. is one of the most important modern plantation crops. The fleshy mesocarp of the fully ripe fruit is rich in valuable edible oils used in the food industry whilst the lipids and fats of both mesocarp and kernel are major components of soaps, emulsifiers and pharmaceuticals. The kernel residues, after commercial extractions, are still useful as animal feed (Hartley, 1967).

The lipid content of the mesocarp continues to rise until the fruit is shed. Fruit that are shed (or bruised) undergo speedy lipolysis and the valuable triglycerides of the mesocarp are hydrolysed to undesirable free fatty acids. For successful economic yields, the harvested bunches should reach the palm oil factory with the maximum number of ripe fruits still attached. The ideal bunches for harvest are, therefore, those containing the highest proportion of fully ripe fruit with a minimum proportion of these in the process of abscission (Chan, Corley, and Seth, 1972).

This paper, which is the first in our study of ripening and abscission in the oil palm, describes a light and electron microscope analysis of the anatomy and the unusual cell separation events of fruit shedding.

MATERIALS AND METHODS

Plant material

Freshly-harvested spikelets of clonal material from the Cameroons or Malaysia were shipped air freight in loose cotton bags enclosed in perforated polythene and boxed in a perforated cardboard container. Spikelets were received in Oxford within 24 h of excision from the parent palm and were then used as experimental material.

Each spikelet was of a known number of days from anthesis and consisted of some 20–40 fruits.

Nomenclature for the different floral parts is based on that of Van Heel, Breure, and Menendez (1987).

Assessment of abscission from freshly-harvested spikelets

Abscission was assessed (a) by the number of fruits that separated naturally after 48 h and (b) the numbers that separated with light pressure. For each separated fruit the position of separation at the fruit base and the presence of the rudimentary androecium or the tepals were recorded (Plate 2B).

Abscission in the field

Shed fruit were collected from the ground beneath marked palms and assessed for the presence of rudimentary androecium or tepals.

Light microscopy

(a) *Separating and non-separating fruit*: Palm fruit were halved longitudinally and fixed in ethanol:glacial acetic acid (70:30, v:v). They were then embedded in polyethylene glycol (PEG) (Water Soluble Wax, Raymond A. Lamb Ltd., London, NW10) and processed according to the method of Pearce and Rutherford (1981). Briefly, fruit were hydrated, infiltrated in a PEG series and embedded in fresh PEG after a 24 h infiltration at 65 °C. Embedded blocks were cooled rapidly and stored. Sec-

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tions (10 μm) were cut on a sledge microtome and the PEG dissolved out in water. Sections were mounted and stained with safranin and fast green by the method of Johansen (Clark, 1981).

(b) *Pre-anthesis inflorescences*: Fixed, longitudinally-halved flowers were embedded in paraffin wax, m.p. 56 °C (BDH Chemicals, Poole, UK). Material was first dehydrated through a series of 2-methyl-propan-2-ol (tertiary butyl alcohol, TBA) and infiltrated with paraffin wax at 60 °C. Embedded tissue was cooled rapidly and stored. Sections (12 μm) were cut on a Cambridge rotary microtome and the paraffin dissolved by immersion in CNP 30 (BDH Chemicals, Poole, UK). Sections were mounted and stained with toluidine blue (Gahan, 1984).

(c) *Localization of polyphenolic compounds*: Cells containing polyphenols are stained deep turquoise with toluidine blue. The presence of polyphenols was further confirmed in longitudinal sections of non-separating fruit which were prepared as described for the pre-anthesis material but stained instead with ferric chloride by the procedure of Gahan (1984).

Scanning electron microscopy

(a) *Longitudinally-cut slices of non-abscinded fruit* included part of the fruit mesocarp below the kernel, the abscission zone and the pedicel. For freshly abscinded ripe fruit the exposed surface at the base of the fruit mesocarp and the exposed surface of the fruit pedicel were prepared. The material was fixed to aluminium stubs with a suspension of carbon in carboxymethyl cellulose solution. The stubs were immersed and frozen in liquid nitrogen and sputter-coated with gold at *c.* −160 °C in the pre-chamber of a Hexland SEM cryo-attachment. The stage of the microscope could be held at *c.* −150 °C or heated to temperatures at which surface ice or frost sublimed. Sections were viewed in a Philips 501 Scanning EM.

(b) *Material from the base of abscinded fruit*, to include the junction with the rudimentary androecium (Plate 6A, B, C), or of the tepals which had separated from the fruit (Plate 6D, E) was fixed directly in 1% aqueous OsO_4 and dehydrated in a graded ethanol series. The material was then dried in a Polaron critical point drying unit and the tissue sputtered with gold and viewed in a Philips 501 Scanning EM.

RESULTS

Abscission of the ripe or ripening oil palm fruit can take place at a number of positions depending upon the conditions and age of the spikelet that bears them.

One site of fruit separation is always at position 1, at the junction of the fruit base with the pedicel (Plate 1A, B; Plate 2A, B). In the field this is generally followed by cell separation in the parenchyma adjacent to the surrounding ring of tissue of the rudimentary androecium, position 2, (Plate 2C, D). This frees the fully ripe fruit so that the rudimentary androecium and all the tepals remain attached to the pedicel tissue in the form of a cup (Plate 1A, B). If a slightly less ripe fruit separates, usually following mechanical disturbances, the line of cell separation may occur outside the ring of the rudimentary androecium at position 3 (Plate 2D), and the fruit is then shed with some or all of the androecial tissue attached. Fruit that are not fully ripe, but have already separated at position 1, can usually be caused to shed by light pressure. The site of cell separation can then be at the base of one of the enclosing whorls of tepals at positions 4 or 5 (Plate 2D). Fruits undergoing separation under these conditions show some of the rudimentary androecium and some or all of the tepals attached, leaving the stump of the pedicel fully exposed. The spiny floral bract and bracteoles (Plate 2A, B) are never shed.

If fruit from freshly-harvested spikelets at different stages of maturation, (that is, days from anthesis) are assessed for the positions of cell separation at shedding (either naturally, or following light pressure at the stigma

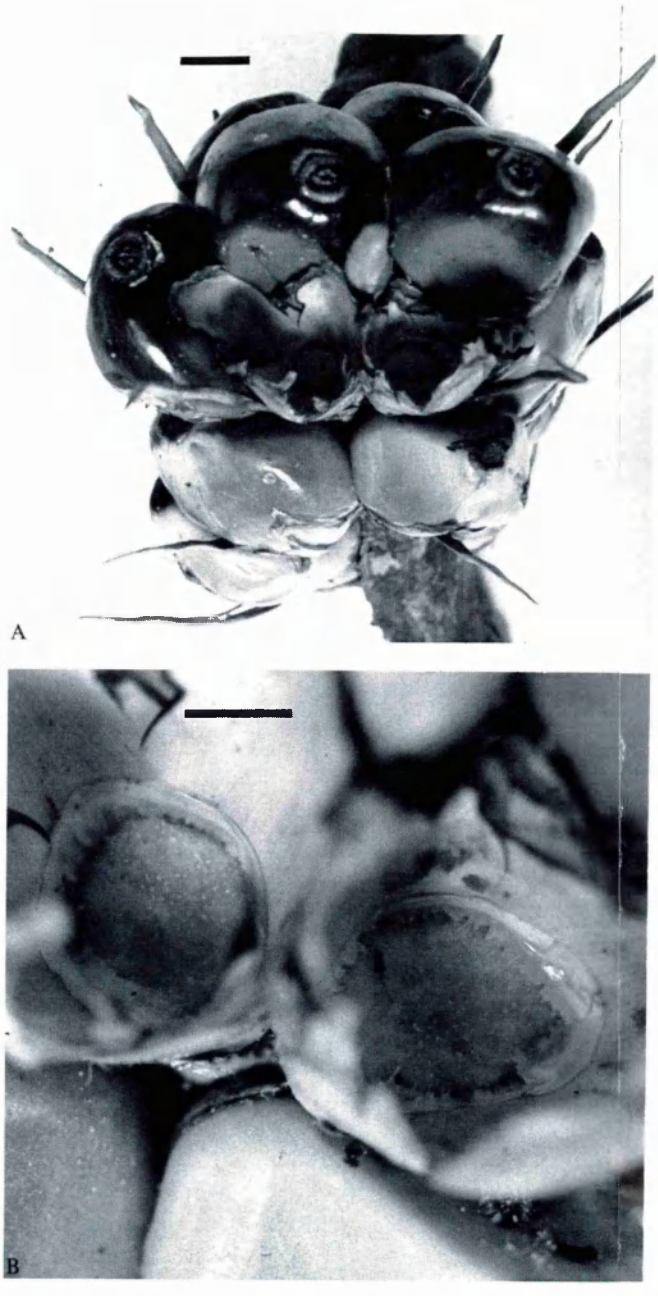

PLATE 1. Oil palm spikelet of clone 90A, 152 d post-anthesis. (A) A ripe fruit showing pedicel surface and remaining tepals and rudimentary androecial ring from two shed fruit. (B) Latter in close-up and enlarged. Scale bars A = 10 mm, B = 5 mm.

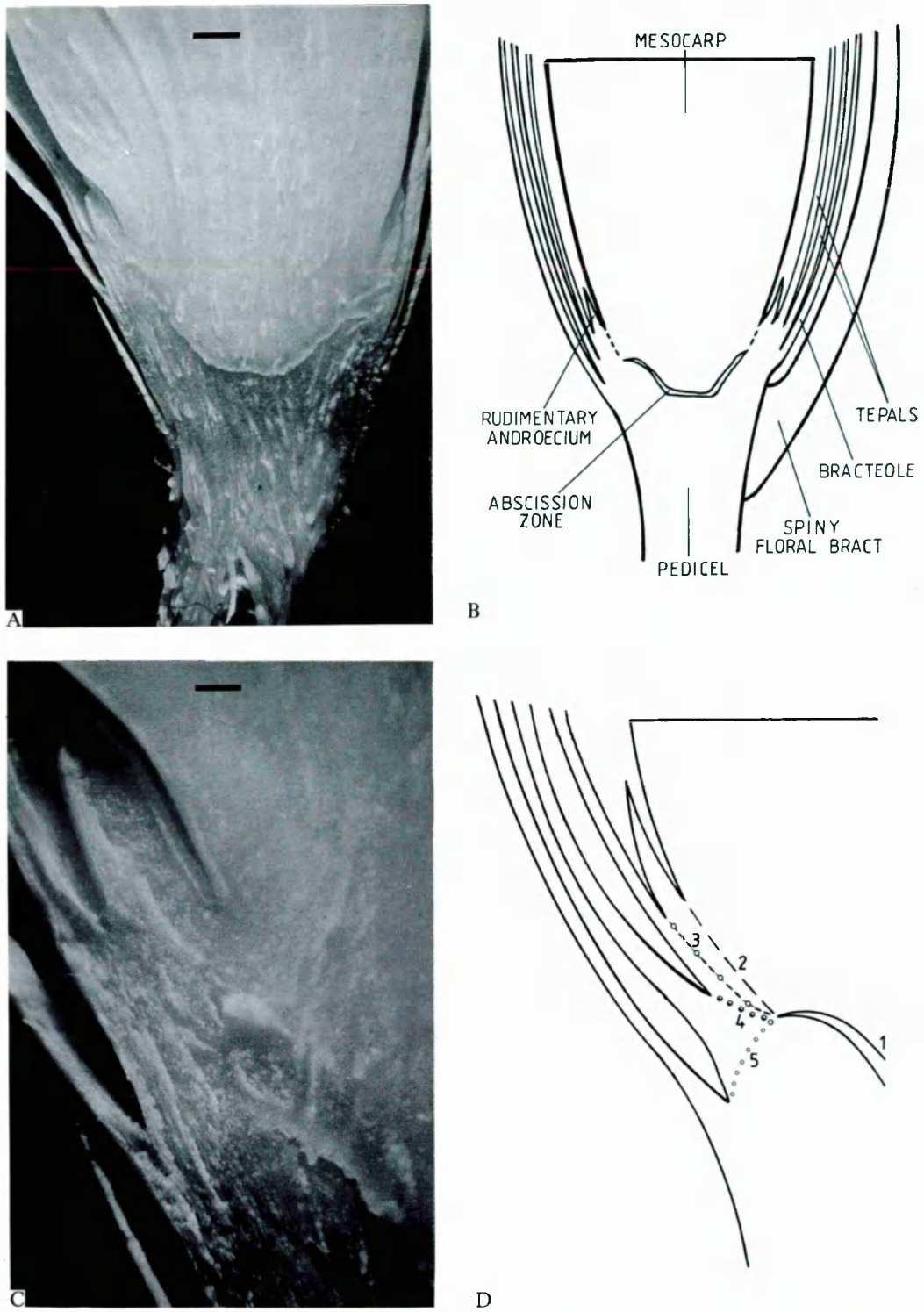

PLATE 2. Longitudinal section of the ripe fruit of clone 926, 149 d post-anthesis. (A, B) In process of abscission, showing position of floral appendages; (C) enlarged close-up of base of rudimentary androecial ring and tepals; (D) the several positions at which cell separation events can occur. Scale bars A = 1 mm, C = 250 μm.

end of the fruit) the propensity to separate at the different sites then depends upon the stage of ripening (Table 1). Unripe fruit will not separate at any position, but ripening fruit will separate at position 1 in association with positions either 4 or 5. Fully ripe fruit always separate at position 1, usually in conjunction with position 2, though sometimes partly with position 3 as found in natural shedding in the field (Table 2). Fruit separation at posi-

TABLE 1. *Percentage fruit abscission from freshly harvested spikelets at different stages of maturation*

All fruits were subjected to light pressure and the numbers and position of separation recorded (for each sample $n = > 200$).

Clone	Days from anthesis	Percentage of total number fruit at each stage		
		Non-Abscinded	Abscission with tepals (position 1+4 or 1+5)	Abscission without tepals (position 1+2 or 1+3)
926	135	100	0	0
	149	48	29	23
	160	0	66	34
	168	0	69	31
90A	136	100	0	0
	147	0	93	7
	160	0	54	46
	168	0	3	97

TABLE 2. *Percentage fruit shed in the field with position of separation and extent of the ring of the rudimentary androecium (RA) remaining attached*

No fruit are shed with adhering tepals, bracteoles or bracts.

	Percentage of fruit abscinded and separation position		
	No RA (Position 1+2)	Partial RA (Position 1+ part 2 and 3)	Complete RA (Position 1+3)
Clone			
926*	84	13	3
975*	95	4	1
90A*	99	1	0
Fruits from seedling palms**	79	13	8

*$n = 100$; **$n = 205$.

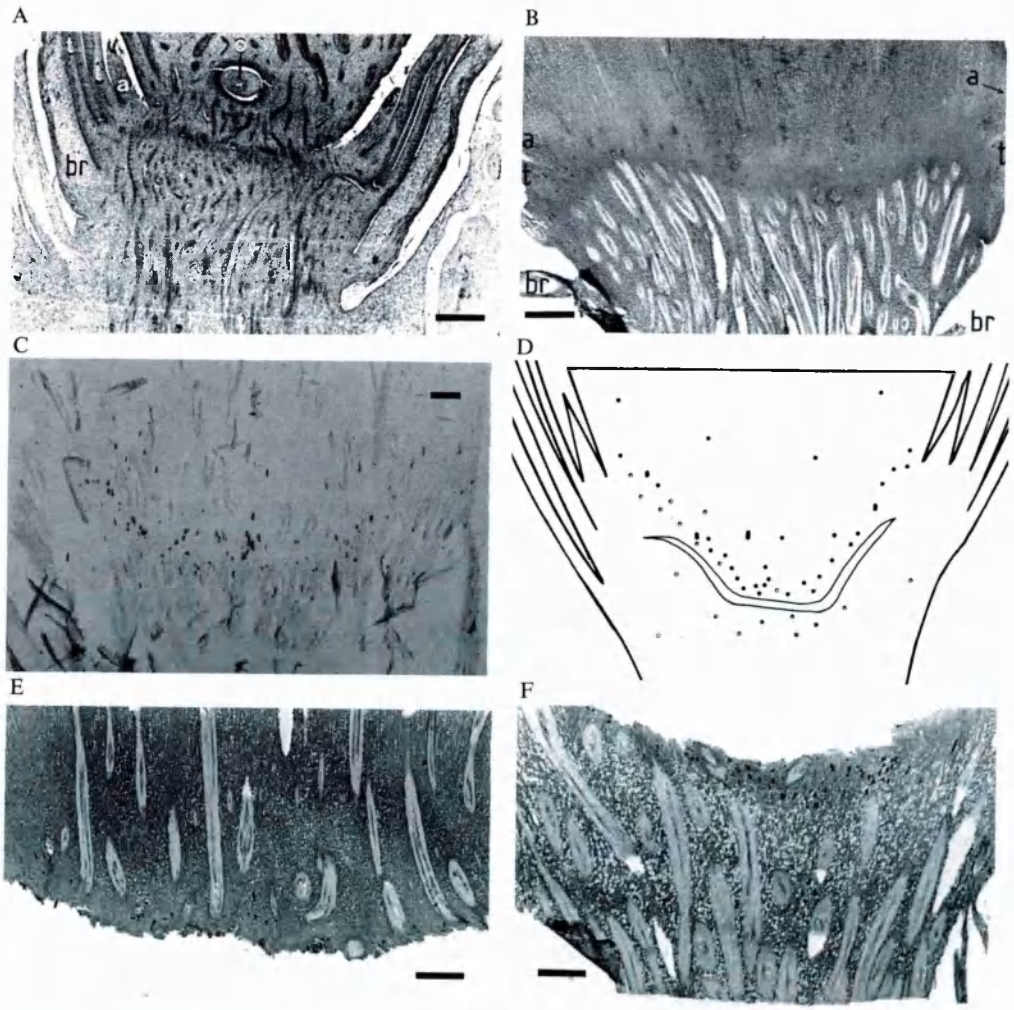

PLATE 3. Light microscope preparations (clone 926) of longitudinal sections of (A) pre-anthesis inflorescence; (B) immature unripe fruit, 81 d post-anthesis; (C) abscission region of nearly ripe fruit, 118 d post-anthesis, stained with ferric chloride to reveal polyphenol-containing cells; (D) diagram showing position of polyphenol-containing cells in relation to the abscission zone; (E) location, in relation to position 1, of densely safranin-staining cells at the fruit base and pedicel region (F) of freshly separated ripe fruit 137 d post-anthesis. o = Ovule, a = rudimentary androecium, t = tepal, br = bracteole. Scale bars A, B, C, E, F = 50 μm. Material preparation (B, E, F) by R. B. Pearce, Department of Plant Sciences, University of Oxford.

tion 1 usually occurs 1 to 3 days earlier than in any of the other positions.

Positions 2 and 3, therefore, afford the sites of final shedding for fruit which have already abscinded at position 1. The later separating positions appear as lines of weakness in an area of tissue that only undergoes appreciable wall loosening after completion of cell separation at the fruit base (position 1).

Differentiation of the abscission zone

(a) *Light microscopy*: Delineation of the abscission zone occurs very early in floral development when it is seen as a band of small cells with dense cytoplasm and prominent nuclei in sections of pre-anthesis fruitlets (Plate 3A). The band of cells widens as the fruit enlarges and the vascular connections, prominent in fruit and pedicel, are conspicuously attenuated at the site of the abscission zone. Even in mature fruit it can be seen that vascular connections directly traversing the abscission zone are rare though many bundles reach the abscission region from both sides (Plate 3). In fruit at 81 d from anthesis, the line of small cells is continuous below the rudimentary androecium and the tepal bases and marks the position at which separations can eventually occur (Plate 3B). The small cells indicate sites of separation when the fruit are shed with their subtending appendages (positions 3, 4, and 5). Fruit at 137 d post-anthesis have loosely adhering walls between fruit and pedicel and sections of tissue are readily disrupted at position 1 during preparation for light microscopy (Plate 3E, F).

Groups of cells, densely staining to safranin, differentiate above and below position 1 as the fruit matures (Plate 3E, F), and specific histochemical stains (see Methods) confirm that they contain polyphenols. However, none of these cells is present amongst those that separate (Plate 3C, D).

(b) *Electron microscopy*: Scanning electron micrographs of a longitudinal section from a non-separating fruit show a specialized structure to the line of cells that comprises the abscission zone (Plate 4A). Higher magnification (Plate 4B, C) reveals the commencement of cell separation in a few pockets of tissue and shows restriction of vascular connections from both fruit and pedicel side at the position of potential separation.

Scans of fruit base and the pedicel surfaces of a freshly abscinded fruit (position 1) show the diversity of type and orientation of the cells that are present (Plate 5A, C). The integrity of the intact walls and the nodular nature of the cell surfaces can be seen at the highest magnifications (Plate 5B, D). Two small clusters of xylem elements are the only cells that are fractured in the region (Plate 5C).

Scans of a freshly separated fruit base cut to include the junction with the ring of the rudimentary androecium (Plate 6A) show that complete cell separation occurs along most of position 2 (Plate 6B). Where the shiny

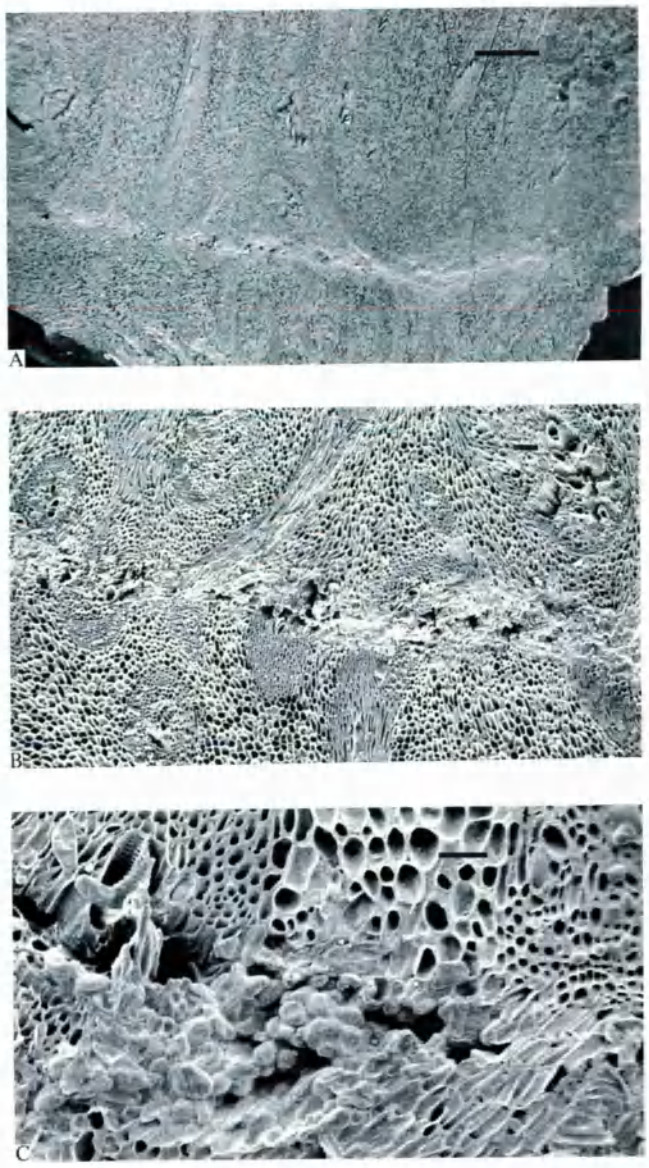

PLATE 4. Scanning electron micrographs of surfaces of frozen longitudinal sections of near-ripe fruit of clone 90A. (A) Line of cell separation between mesocarp and pedicel; (B) groups of separating cells and arrangement of vascular bundles; (C) intact but separated cells of the zone with fractured xylem elements. Scale bars A = 1 mm; B = 100 μm. C = 50 μm. Material preparation and photographs by J. A. Sargent, Electron Microscopy Consultancy, Mendota House, Islip, Oxford OX5 2SB.

mesocarp joins the androecium, however, several rows of cells are clearly fractured (Plate 6C) indicating cell breakage, rather than cell separation.

Scans at the base of a fruit-tepal junction (position 3) show only intact cell separation and no cell fracturing (Plate 6D, E).

DISCUSSION

In most species the abscission of leaves and fruits occurs across a plate of zone cells which are positionally differentiated early in the development of the organ. The active

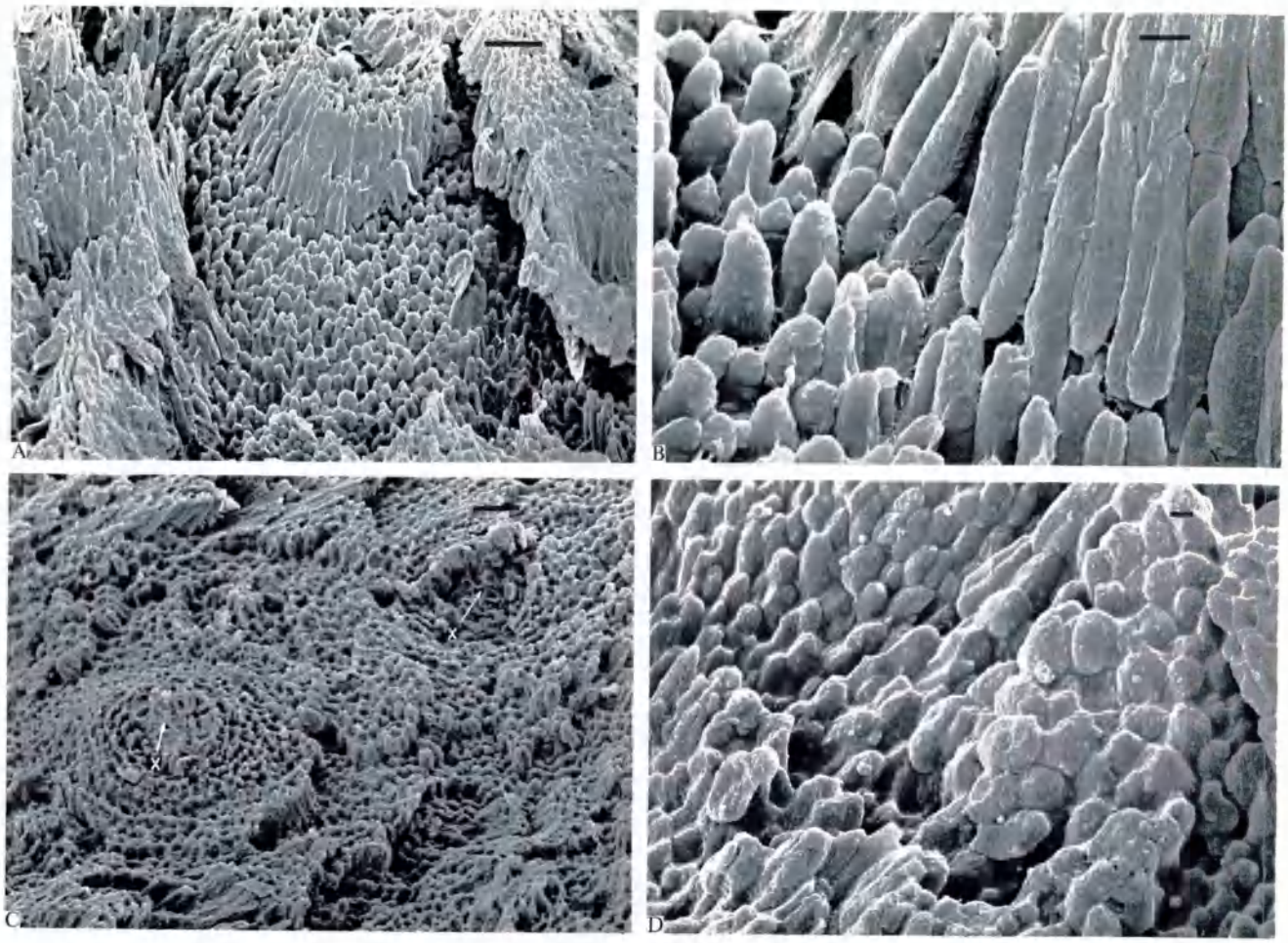

PLATE 5. Scanning electron micrographs of the fruit base (A, B) and pedicel (C, D) surfaces of a freshly abscinded and frozen ripe fruit of clone 90A showing intact separated cells with nodular adhesions to the wall surfaces (B, D) and the presence of an extra-cellular wall coating particularly evident on the pedicel surface (D). x = Fractured xylem or fibre elements. Scale bars A, C = 50 μm; B, D = 10 μm. Material preparation and photographs by J. A. Sargent, Electron Microscopy Consultancy, Mendota House, Islip, Oxford OX5 2SB.

cell separation process at fruit ripening depends upon the secretion of specific enzymes and the loosening of chemical bonds in the middle lamella region between them so that the fruit is shed as a single-stage event (Addicott, 1982; Sexton and Roberts, 1982; Osborne, 1989).

The shedding of ripe fruit of the oil palm is unusual in its bi-phasic progression. Only one report, also for monocotyledons, has described a bi-phasic cell separation sequence in fruit abscission. The separation described for *Avena fatua* and certain other Gramineae, however, contrasts with that of the oil palm for although it occurs in two phases these are separated developmentally and temporally by several weeks. The first phase results in loss of adhesion of the walls of zone cells, except at the plasmodesmata, whilst the second phase involves wall dissociation at the attached plasmodesmatal domains with an ensuing shedding of the fruit (Sargent, Osborne, and Dunford, 1984).

In the oil palm, an abscission event, in the true sense of a regulated cell separation, is induced first at a predetermined and positionally differentiated locus at the junction of the fruit and its pedicel (position 1). This is only a prelude to the final separation process. At the stage when all cellular connections between the fruit base and pedicel are disrupted, the ripe fruit is still firmly held within the surrounding cup of the rudimentary androecial ring, the tepals, bracteoles and spiny floral bract. Full cell communication is initially still retained between the pedicel and the bases of these appendages, presumably permitting the transfer of water and solutes between them (Plate 2). All further cell separations take place in an area bounded by the perimeter region of the pedicel and the fleshy bases of the appendages. It would appear that the cells in this whole perimeter area undergo a change in the tenacity of wall adhesion when the fruit is ripe and the tissue tensions exerted while the fruit is retained in the cup eventually determine the line of weakness that will finally release the fruit. In the field (Table 2), this generally occurs at the inner face of the rudimentary androecial ring (position 2) so that a completely naked fruit is shed. A few

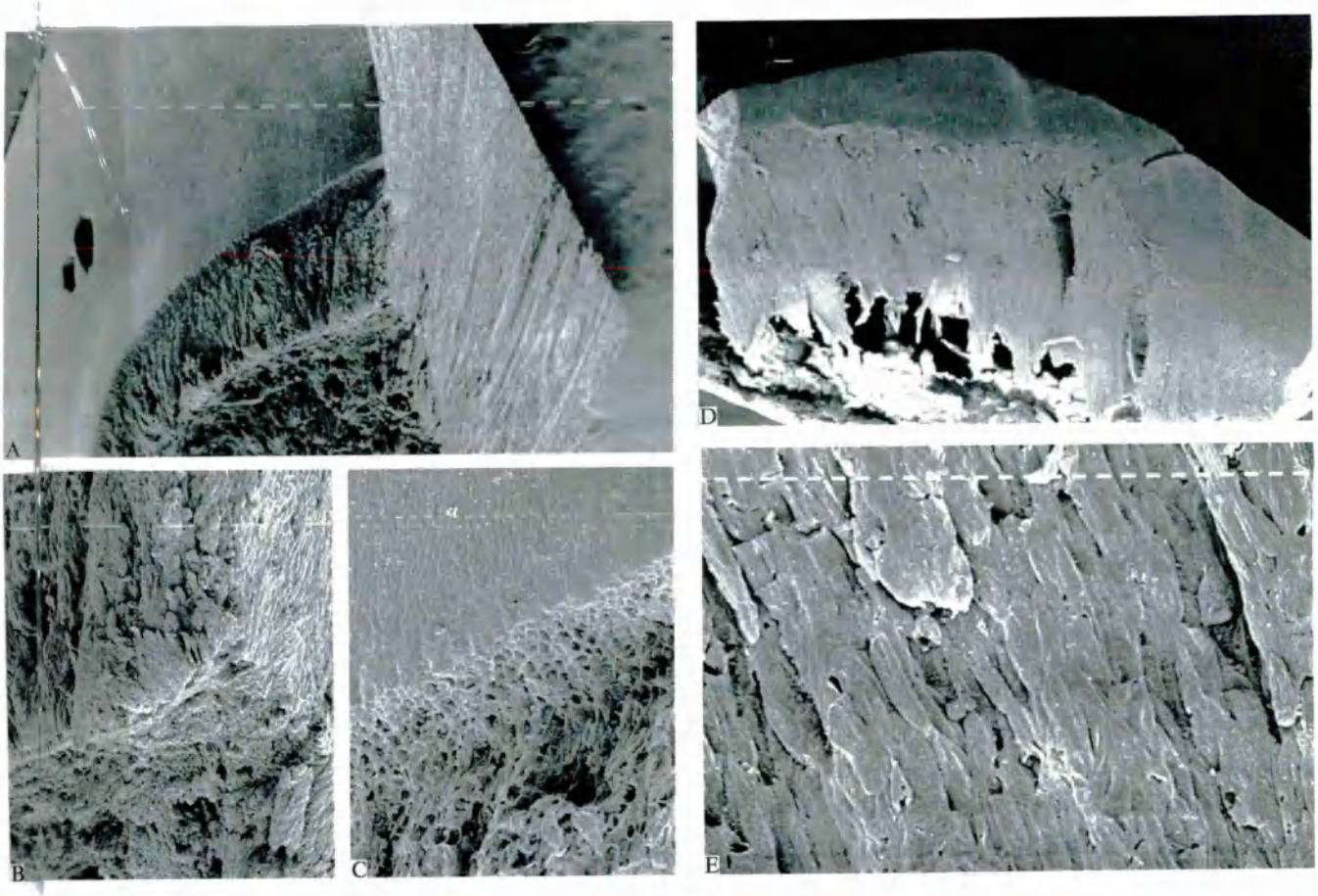

PLATE 6. Scanning electron micrographs of the freshly-separated face of a 149 d post-anthesis ripe fruit of clone 90A prepared by OsO_4 fixation and critical point drying to show (A) separation positions 1 and 2; (B) the intact cells at the separation face of the fruit base (position 1) and the mesocarp junction with the rudimentary androecial ring (position 2); (C) the fractured cells present at the distal region of the separation at position 2 immediately adjacent to the shiny epidermis (exocarp) of the fruit wall; (D, E) base of separated tepal to show intact cells at position 4. Scale bars A, B = 100 μm; C = 10 μm (large scale bar = 100 μm); D = 100 μm; E = 10 μm. (The two perforations in the fruit wall of A are from forceps.) Material preparation and photographs by T. S. King, the Open University E.M. Suite, Milton Keynes, MK7 6AA.

shed fruit are found with only parts of the androecial ring attached (some separation has occurred at position 3) but it is rare to find fruit with complete rings. Tepals, bracteoles and the large spiny bract always remain upon the palm (Plate 1).

On harvested spikelets, where tissue tensions and water relations may well be different, only those with already near-to-shedding fruit (Table 1, Clone 90A, 168 d post-anthesis) will separate with none or only part of the rudimentary androecial ring attached. More usually, when subjected to light pressure, fruit are shed with the ring and with some or all of the tepals, though bracteoles and the floral bract always remain on the spikelet. Unless separation first occurs at position 1, however, no shedding will take place. Fruit that are unripe do not undergo cell separation, either at position 1 or at other positions and are, as a consequence, never shed (Table 1).

The abscission zone cells at the base of the ovary are already differentiated in the pre-anthesis inflorescence (Plate 3A) though at this stage there are no anatomical features to distinguish the eventual separations at positions 2, 3, 4, or 5. At no time are complete vascular bundles continuous across the abscission zone (Plate 3B). As the unripe fruit matures, phenolic-containing cells are differentiated on either side of (but not within) the zone and are readily visualized with Johansen's stain (Plate 3C, D). The phenolic content of the cells in unfixed, freshly sectioned, tissue was confirmed by Professor A.M. Mayer using the nitrous acid test (Gahan, 1984) and showed well developed groups of 1–3 cells above and below the abscission zone (although not within the zone). Other groups of phenolic cells are scattered in the mesocarp tissue below the exocarp and around the vascular bundles. The role that these cells may play during development remains unknown, but after abscission those below the exposed pedicel surface may afford protection against pathogenic invasions as suggested for phenol cells in other species (Scheffer and Cowling, 1966; Rhodes, 1985).

The differentiation of the abscission zone can be seen most clearly in the scanning electron micrographs (Plates

4, 5). In the longitudinal sections of near-ripe fruit, certain of the zone cells are already loosely associated at position 1 whilst others are still adhering along their whole wall surface at the fruit-base pedicel junction. The few xylem and fibre elements that traverse the zone become fractured during abscission and these regions are visible as small spikes on the separated pedicel surface (Plate 5C). They are also noticeable as white flecks on the pedicels of freshly separated fruit (Plate 1B). The exposed surfaces of the just-separated cells of the fruit base and pedicel in freshly abscinded fruit (position 1) are of special interest. Nodular structures are apparent on the surfaces of the fruit base but are considerably more evident on the surface of the pedicel cells where they appear in association with a coating of amorphous material that may be pectinaceous in nature (Plate 5). Whether or not these surface characters are the result of dissolution of the middle lamellae by enzymes secreted during abscission remains to be determined, but they are commonly observed in intercellular cell separations in other species (Carr and Carr, 1975) and pectinaceous 'beads' have been reported on the surface of opposing cells in graft unions (Jeffree and Yeoman, 1983).

Cell separations at positions 2, 3, 4, and 5 (Plate 6) do not show the same wall surface configurations found on cells in position 1. However, with the exception of the distal edge of the rudimentary androecial ring with the shiny fruit exocarp, (where the cells are clearly fractured (Plate 6C)), intact cell walls are visible throughout the area in which the rudimentary androecial ring separates from the fruit base (Plate 6B) and where the tepal bases separate from the pedicel (Plate 6D, E).

Since separation in these positions does not occur until after abscission has been initiated at position 1 it is tempting to speculate that control of separation at positions 2, 3, 4, and 5 lies with an activation of, and enzymic secretion from, the zone cells at position 1. The subsequent diffusion of exo-glucanhydrolases (pectinases, cellulases or polygalacturonases) through the apoplast may lead to the eventual loosening of the enzyme-susceptible middle lamellae and walls in the tissue differentiated at the peripheral margin of the pedicel where the bases of the androecial ring and the tepals join (Plate 2C). This unusual abscission process of the oil palm in which the fruit remains loosely attached for a period before final shedding may well be associated with the behaviour of the tropical birds, fruit bats and climbing animals that remove the partially attached fruit and so act as dispersal agents in the palm's native habitats in West Africa.

Hormonal controls and biochemical changes during oil palm fruit ripening and abscission are the subject of our next publications.

ACKNOWLEDGEMENTS

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LIPASE ACTIVITY IN RIPENING AND MATURE FRUIT OF THE OIL PALM. STABILITY *IN VIVO* AND *IN VITRO*

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Key Word Index—*Elaeis guineensis*; Palmae; lipases; rapid assay; enzyme stability; development; chilling injury.

Abstract—A quick and reliable method is described for the extraction and assay of active lipase from ripening fruits of the oil palm using glycerol tri[1-^{14}C]oleate as substrate. The enzyme is lipophilic and is readily inactivated *in vitro* at temperatures above 0°. *In vivo*, the enzyme activity increases as ripening proceeds. The lipase of ripe fruit is particularly sensitive to chilling inactivation (8°) with no restoration of activity at 25° but is stable for at least 8 days in fruit held continuously at 20° or for short periods (30 min) at 45°. Extended periods (10 hr) at 45° or 30 min at 55° are sufficient to irreversibly inactivate the enzyme in intact fruit. Enzyme induction is coincident with the onset of fruit ripening and would appear to be the ripening-associated expression of a new gene activity.

INTRODUCTION

The ripe fruit of the oil palm is a rich source of unsaturated and saturated lipid and important in the food industry worldwide. The mesocarp lipid of elite clones can reach 60% by fresh weight of low melting point triacylglycerides primarily of oleic and palmitic acids [1] while palm-kernel oil from the endosperm contains other storage lipids of high lauric acid content.

Much is known of the chemistry, stability and degradation products of the mesocarp lipids (palm oil) during storage [2] and the metabolism of the kernel reserves has received considerable study during germination of the seed [3]. Relatively little is known of the factors that regulate the development of lipid synthesis in the mesocarp or of the factors initiating conversion of the accumulated triglycerides to the economically undesirable free fatty acids after the fruit is fully ripe. This loss of mesocarp lipid becomes significant only at the stage of fruit shedding so that on the plantations factory processing of the ripe bunches is a daily event, and preferably started within 12 hr of harvesting, to ensure the highest and best quality yields of palm oil with the lowest possible content of free fatty acids [4].

The distribution of lipases in the oil palm appears limited to specific tissues. Whereas an active lipase has been demonstrated in the shoot of germinating seedings, no activity has been detected in either the kernel or the seedling haustorium [5]. Although the existence of active lipases in the mesocarp of the ripe fruit was long suspected [6] there has been some uncertainty as to its presence and debate as to its origin. Some investigations failed to detect either the enzyme or an inhibitor of the enzyme upon extraction of the fruit [7], whilst others suggested that lipid degradation at ripeness was primarily attributable to lipases originating from contaminating yeasts or other invading micro-organisms [8]. That an active lipase is indeed present in the flesh of the ripe mesocarp has, however, now been convincingly demonstrated [9].

As part of our study on fruit ripening and abscission in the oil palm we have followed the development of a lipase activity which is linked to the progress of lipid accumulation in the mesocarp, and now describe experiments which provide information on temperature factors regulating the stability of the enzyme *in vitro* and *in vivo*, using a rapid test assay to detect this activity in mesocarp preparations of the fruit.

RESULTS

Characteristics of the mesocarp lipase

Lipase activity was assessed in fruits at different stages of development from 17 days after anthesis until shedding. Activity was present in mesocarp powders or in phosphate buffer homogenates of ripening and ripe fruit. Highest activity was present in the ripest fruit coincident with the abscission of the fruit base from the pedicel. Activity rose from background values in the unripe fruit to just detectable in fruit that were first synthesizing carotene and lipid (*ca* 125–130 days after anthesis, Fig. 1).

In the aqueous extracts, activity was present in all three layers formed when the homogenate was centrifuged, with greatest being in the upper lipid phase. In the middle aqueous layer the lipase was probably associated with the presence of small lipid globules and micelles as activity was progressively reduced when this aqueous layer was filtered through 0.45 μm, 0.2 μm membranes, and was not detectable after filtration through 0.02 μm pore size (Acrodisc, Gelman Science). Lipase activity of this aqueous layer was always lower than that of the equivalent of frozen mesocarp powder (Fig. 2). Some activity remained in the pellet, probably associated with trapped lipid. For 30 min incubations the activity of both aqueous extracts and mesocarp powder decreased as the temperature of the assay was increased from 18 to 35° (Fig. 2) and this was assumed to be associated with a greater instability of the lipid micelles at these temperatures.

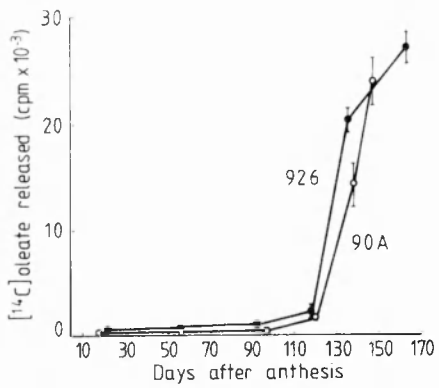

Fig. 1. Increase in lipase activity of mesocarp powders prepared from developing fruits of oil palm clones 90A and 926. Carotene formation indicative of ripening starts to occur between *ca* 120 and 125 days after anthesis. For sample preparation and assay see Experimental.

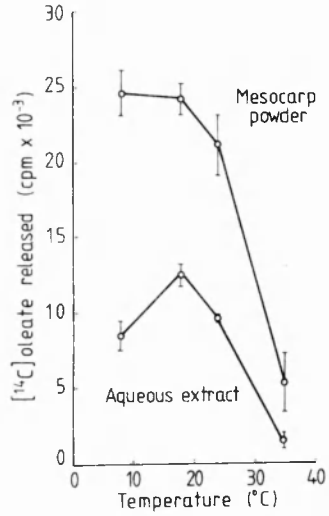

Fig. 2. Comparison of lipase activity in mesocarp powders and aqueous extracts from ripe fruits of clone 90A 163 days after anthesis. For sample preparation and assay see Experimental.

In vivo, the lipase activity of fruit was fully retained when spikelets were kept at room temperature (above 20°) for up to 8 days. Similarly, no decline in lipase activity occurred when freshly harvested spikelets were frozen in liquid nitrogen and kept at −17° for 7 days (Table 1). However, the enzyme was unstable after cell disruption. Whereas the freshly prepared mesocarp powders remained fully active if maintained at −17°, lipase activity decreased to 69% of the original value after one hour if the powders were kept on ice and loss was even more rapid at 25°, decreasing to 33% of the original value after 15 min (Table 1). A comparison of detergents Tween 20, Tween 80 or Triton X-100 in the extraction medium and cocktail showed Tween 20 to be superior for obtaining and retaining activity. Attempts to improve extractibility and stability of the enzyme by increasing ionic strength were unsuccessful. At 1.0 M NaCl, activity was reduced by *ca* 30% in the lipid layer and by *ca* 50% by 200 mM in the aqueous layer. Additions of Ca^{2+}, papain or PMSF also failed to enhance or prolong the lipase activity in mesocarp powders or in lipid or aqueous layers of the homogenates.

In assays of mesocarp powder, radioactivity released as the [^{14}C]oleate hydrolysis product increases with time. The reaction is near linear for at least 20 min at 18° (Fig. 3A) and the rate increases with increasing concentration of the glycerol tri[1-^{14}C]oleate substrate in the medium (Fig. 3B). Maximum product release per 30 min assay occurs at *ca* 6 mg mesocarp powder per assay and no inhibition is observed with increasing sample weight (Fig. 3C). These are characteristics of enzymic hydrolysis rather than autocatalysis. Highest activity was observed under our assay conditions at pH 6.5–6.8 (Fig. 3D). In a comparison of substrate specificity of their oil palm lipase, Abigor, *et al.*, [9] tested a number of lipid substrates. They found that glycerol tri-oleate was hydrolysed with an 83% efficiency of that of native palm oil but oil of another palm (coconut) and olive oil were poorly hydrolysed (3 and 33% respectively). Hence the procedures for extraction and assay that we have adopted provide for a highly specific yet sensitive, speedy and reproducible means of determining the activity of lipase from fruit mesocarp.

Effect of low and high temperatures on the lipase enzyme in vivo

When fresh spikelets or individual fruit were cooled in the refrigerator (8°) for different periods and mesocarp powders or aqueous extracts subsequently prepared, the lipase activity decreased progressively with time. Clone 926 was considerably less sensitive to 8 hr of chilling than clone 90A; the ripest fruit of 90A showing the highest loss of lipase (Table 2). Activity could not be restored in either clone by subsequently returning the fruit to room temperature. In fact, the continued maintenance of the treated fruit at 25° resulted in further loss of lipase activity (not shown). The reduction of lipase activity with continued cooling occurs soonest in the ripest fruit (Fig. 4) and accords with the greater chilling sensitivity in fruits of other species as ripening proceeds.

Considerable reduction in lipase activity occurred when fruit were exposed to temperatures above 45° for a short period (55°, 0.5 hr) before the mesocarp powders were prepared (Table 3a), although there was no loss when the fruit held at 45° for only 0.5 hr were re-tested after keeping at 25° for a further 24 hr (Table 3a). In fruit held for longer periods at 45° (2, 4 or 10 hr), lipase activity was essentially abolished and could not be restored (Table 3b). Fruit of both clones showed equal sensitivity to these elevated temperatures.

Changes in mesocarp lipase activity with maturation of the fruit.

Mesocarp powders prepared from fruit ranging from 17 to 164 days after anthesis showed barely detectable lipase activity until 90–95 days post-anthesis, but from 120 days onwards, activity increased steeply (Fig. 1). Similar results were obtained for the two different clones, 90A and 926. This rise in activity corresponds closely with the onset of fruit ripening and would appear to be coincident with the start of synthesis of acyclic carotenoids (α- and β-carotene). This development of orange

Table 1. Stability of the lipase enzyme *in vivo* and *in vitro* with time and storage conditions

	[^{14}C]oleate released (cpm/10 mg mesocarp powder/30 min)
(a) Fruit on spikelet for:	
i) 4 days at 25°	22 179 ± 725
ii) 8 " " 25°	29 597 ± 932
iii) 7 " " −17°	25 295 ± 1 113
(b) Mesocarp powders:	
i) Immediately after grinding with liquid nitrogen	23 687 ± 2 412
ii) 1 hr on ice	16 316 ± 1 621
iii) 2 min at 25°	16 480 ± 2 426
iv) 15 " " 25°	7 787 ± 1 497
v) 5 days at −17°	22 228 ± 1 135

Assayed as [^{14}C]oleate released from glycerol tri[1-^{14}C]oleate by mesocarp powders (see Methods). Clone 90A 149 days after anthesis. (a) Mesocarp powders prepared from fruits kept on spikelets. (b) Prepared mesocarp powders. Values, mean of triplicates ± s.d.

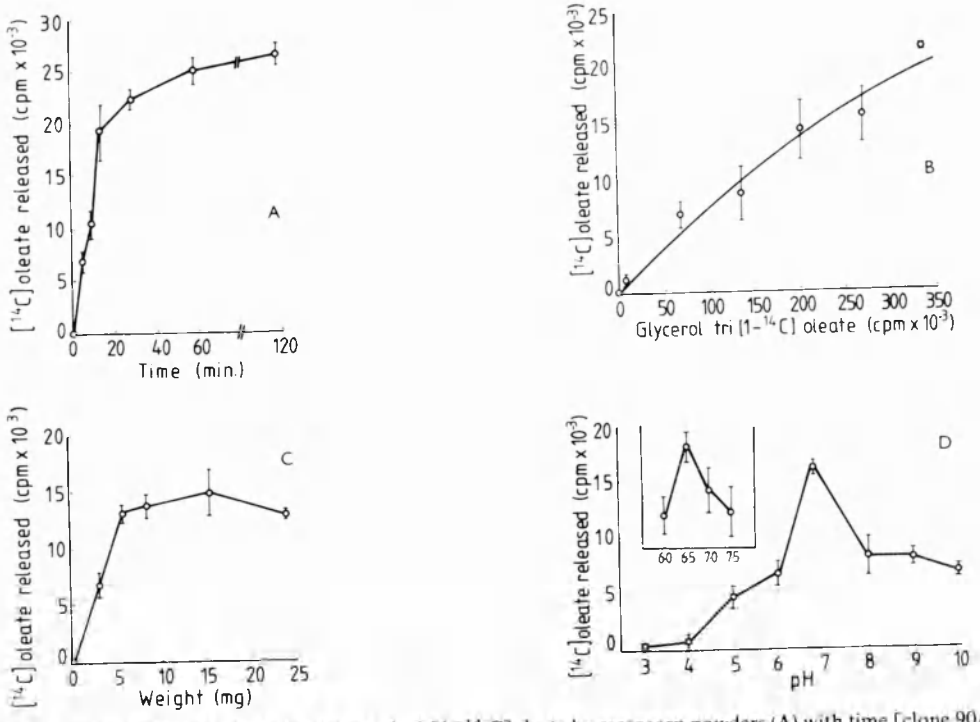

Fig. 3. Kinetics of lipase hydrolysis of glycerol tri-[1-^{14}C]oleate by mesocarp powders (A) with time [clone 90A 149 days after anthesis]; (B) with increasing substrate concentration [clone 926 154 days after anthesis]; (c) with increasing sample weight [clone 90A 164 days after anthesis]; (D) with assay pH [clone 90A 149 days after anthesis—insert, 164 days after anthesis]. Assay time for B–D, 30 min. For A, B, D, weighed mesocarp powders 6–10 mg. For sample preparation and assay see Experimental.

pigmentation has been shown to coincide with the attainment of maximum fruit size and the initiation of lipid deposition [10, 11]—also, ripening or lipid accumulation in fruits is not initiated before *ca* 120 days from anthesis. Thereafter, lipid accumulation is rapid (×4 in 7 days, see [1]) and mirrors our Fig. 1. This corresponds with our observation of the first appearance of a lipid layer in aqueous homogenates of mesocarp at this stage of development. Lipid accumulation and the rise of lipase appear therefore to be closely linked in time.

In any ripening spikelet, we find that lipase activity is highest in the terminal fruit with decreasing levels towards the base of the spikelet. This sequence too, follows that of fruit ripening. In view of the coincident appear-

Table 2. Sensitivity of lipase in fruit of clones 90A and 926 to chilling (8°) for 8 hr

Days after anthesis		[1-^{14}C]Oleate released (cpm/10 mg mesocarp powder/30 min) 0 hr	After 8 hr at 8°
Clone 90A	148	17 885 ± 206	20 678 ± 475
	161	17 213 ± 641	5 712 ± 1 412
	175	16 772 ± 3 707	3 788 ± 70
Clone 926	154	20 904 ± 2 759	16 324 ± 1 903
	161	17 903 ± 981	10 997 ± 1 648
	175	20 263 ± 886	15 309 ± 2 998

*Values, mean of triplicates ± s.d.

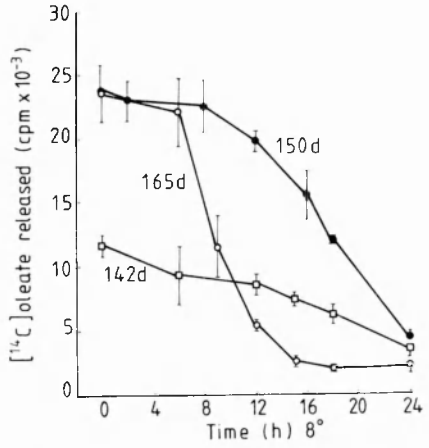

Fig. 4. Loss of lipase activity in fruits of clone 90A at different stages of ripeness (days after anthesis) when maintained at 8°. For sample preparation and assay see Experimental.

ance of lipid and lipase and the high lipophilicity of the enzyme it could be questioned whether the absence of the appropriate level of endogenous lipid could be the cause of a failure to obtain an active lipase from unripe fruit. If this were so, then the addition of the lipid-rich, but lipase-inactivated, mesocarp tissue from fully ripe fruit to samples of unripe fruit before preparation of the mesocarp powders should provide the appropriately lipid-enhanced environment for detecting enzyme activity if any lipase were present in unripe fruit. It is clear, however, from the results of the experiments set out in Table 4, that the addition of lipid present in the tissue of lipase-inactivated ripe fruit neither enhances activity from unripe mesocarp nor reduces the activity from ripe mesocarp. One must conclude, therefore, that an active lipase enzyme is indeed absent from unripe fruit.

DISCUSSION

Although difficulties have been experienced in past attempts to detect lipases in the mesocarp of ripening oil palm fruits, it seems that part of the problem has been associated with the instability of the extracted enzyme (which is readily inactivated at temperatures above freezing) and the necessity for maintaining a stable micellar structure in the assay medium. Highest activity was consistently obtained by us with mesocarp powders or with the lipid layers of aqueous homogenates, rather than in the aqueous phases, indicating the greater stability of the enzyme in a hydrophobic environment.

From phosphate buffer homogenates, Abigor *et al.* [9] describe the optimum temperature for their lipid phase mesocarp lipase at a considerably higher temperature (30°) than we have used, with linearity of reaction for 20 min using palm oil as the substrate. They found no detectable activity in their aqueous layer, possibly through an absence of micelles, and concluded, as we do, that the lipase retains its hydrolytic function in a lipid environment. Although they were able to remove lipid by ether extraction of the lipid phase and retain activity in

Table 3. Lipase activity in mesocarp powders following exposure of the intact fruit to elevated temperatures

Treatment	[^{14}C]Oleate (cpm/30 min) released from glycerol tri[1 − ^{14}C]oleate	
	Clone 90A 175d	Clone 926 176d
(a) Fruit held at 25°	28 350 ± 2 285	25 461 ± 250
0.5 hr 35°	27 543 ± 1 746	28 757 ± 537
" 45°	24 003 + 446	23 461 ± 2 637
" 55°	296 ± 100	317 ± 41
0.5 hr 45° + 24 hr 25°	28 957 ± 1 709	26 888 ± 2 155
0.5 hr 55° " "	2 166 ± 200	176 ± 38
	Clone 90A 161d	Clone 926 176d
(b) Fruit held at 25°	19 839 ± 1 726	20 425 ± 1 311
0.5 hr 45°	19 413 ± 1 707	23 461 ± 2 637
2.0 hr "	235 ± 57	517 ± 185
4.0 hr "	208 ± 40	391 ± 157
10.0 hr "	95 ± 19	64 ± 13

Values, mean of triplicates ± s.d.

Table 4. Effect of additional mesocarp lipid on the lipase activity of mesocarp powders prepared from immature and ripening fruit

Days after anthesis	[^{14}C]Oleate released from glycerol tri[1-^{14}C]oleate (cpm/30 min)		
	Mesocarp powder (5 mg)	Mesocarp powder (10 mg)	Mesocarp powder (5 mg) with lipase-inactive mesocarp powder (5 mg)
23 (unripe)	—	991 ± 88	468 ± 90
63 (unripe)	—	251 ± 106	270 ± 89
125 (ripening)	—	15 536 ± 1 450	10 341 ± 1 310
142 (ripening)	10 717 ± 735	11 680 ± 891	10 378 ± 3 747
165 (ripe)	20 182 ± 2 283	20 420 ± 1 833	17 362 ± 1 448

Mesocarp powders from tissue of fruits of clone 90A were assayed at different stages of development from 23 days after anthesis and compared with powders prepared from a mixed tissue which included high lipid-containing mesocarp tissue from lipase-inactivated ripe fruit. To obtain the lipase-inactivated mesocarp (clone 90A 165 days after anthesis) fruit were first exposed to 45° for 10 hr. Values, mean of triplicates ± s.d. Lipase-inactivated mesocarp powder alone (10 mg) 42 ± 13 cpm.

the remaining protein we have found up to 50% loss of activity following this procedure.

Like many tropical fruits, the ripe or ripening oil palm is chilling sensitive and temperatures below that of chilling damage (8°) will inactivate certain enzymes. The lipase of clone 90A is particularly susceptible to such inactivation (Table 2, Fig. 4) with no reactivation on return to 25°. Phase changes in the fruit membranes below the critical chilling temperature may be the cause of this loss of extractable lipase activity and may be a further cause for the failure of some experimenters to isolate a lipase from pre-cooled material. Inactivation occurs too, at elevated temperatures (0.5 hr at 55° and 2 hr at 45°, Table 3) and also without complete reactivation at 25°. In addition, the mesocarp of cooled or heated, ripe fruit changes from bright orange to dull-brown colour, perhaps due to the activity of polyphenoloxidases which we have already demonstrated in certain cells of the mesocarp [12], although factors inhibitory to lipase activity appear not to be produced (Table 4). However, if the fresh fruit are flash-cooled in liquid N_2 or the mesocarp powders prepared from the fruit are kept at −17°, lipase activity is fully maintained indicating that the enzyme itself is not subject to chilling damage (Table 1). Changes in enzyme activity of fruits on chilling *in vivo* are not uncommon and may increase or decrease. Of the few examples of decreases in the literature most show recovery on return of the fruit to ambient temperature. The catalase of cucumber, for example, is depressed by half after 48 hr at 5° but is restored to 75% of original activity by 48 hr at 25° [15]. It is not clear, however, to what extent this recovery is attributable to new synthesis. Oil palm lipase is possibly unusual in the irreversible loss of activity *in vivo*.

The development of an active lipase with the onset of visible carotene formation and lipid deposition in the fruit mesocarp may be highly significant. The enzyme *in vivo* could be involved in lipid synthesis and deposition. Alternatively, it could be fully sequestered from the sites of synthesis and only active in triglyceride hydrolysis when the fruit is fully ripe and partially shed, e.g. when the base of the fruit has separated from the pedicel but the fruit is still retained by the bracteole bases and the intact rudimentary androecium [12]. Removal of fruit or excision of the bunch leads at once to cessation of lipid synthesis [4] and bruising of the fruit has long been known to accelerate triglyceride conversion to free fatty acids. Both responses may represent the loss of internal compartmentation in the fragile cytoplasmic organization of the ripe mesocarp. It has proved extremely difficult to obtain electron micrographs of ripe fruit in which organelle membranes are intact. However, of the very many that we have prepared of the mid-region of fruit (the position that we normally sample for lipase assays at all stages of development from unripe to shedding) we have yet to observe any contamination of the mesocarp by micro-organisms. This indicates that the production of free fatty acids in the ripe fruit must be primarily controlled by an endogenous lipase whose hydrolytic function is activated by shedding or by wounding.

Whether the conditions that lead to the lipase hydrolysis of accumulated mesocarp triglycerides are first signalled by abscission of the fruit base from the pedicel or by the later cell separation of the enclosing bracteoles remains to be discovered. Field studies indicate that lipid levels are maintained until fruit is actually shed [13]. However, the increase in free fatty acids reported for fruits which became loose and readily detached by slight manipulation [14] would indicate that it is the separation of the fruit/pedicel abscission zone which is the initiating event. As fruit that are parthenocarpic show a similar degradation of lipid to free fatty acids as that of seeded fruit the signal for hydrolysis is evidently not provided from a developing embryo.

We have shown that during development a rise in lipase is initiated with the onset of ripening and is not dependent upon a limiting level of lipid in the cells for the preparation of active extracts. It would appear, therefore, that this lipase is one of the inducible and ripening-associated gene expressions in the oil palm fruit. It is tempting to speculate that the enzyme plays a role in lipid synthesis, perhaps in transesterification [16] during

ripening. It is already known that the fatty acid composition of the triacyl glycerides undergoes progressive change [1]. Lipase and lipid production may therefore be linked responses that could be induced by the same initiating or transduction signal(s).

EXPERIMENTAL

Plant material. Freshly harvested spikelets of clonal material (*Elaeis guineensis* Jaeq.) from Malaysia were shipped air-freight in loose cotton bags enclosed in perforated polythene and boxed in a perforated cardboard container. At no time during transit did the temp. of the fruits fall below 12° nor did it rise above that experienced by fruit in the field (maximum 40°). Spikelets were received in Oxford within 24 hr of excision from the parent palm and were used as the experimental material. Each spikelet was a known number of days from anthesis and consisted of 20–40 fruits.

Pre-treatment of fruit at different temperatures. For cooling experiments, spikelets or individual fruits were placed in cotton bags inside a plastic bag and kept in either a low temp. deep freeze (−17°) or the refrigerator at 8°. For treatments above room temp., samples of individual fruits were enclosed in plastic bags containing lead weights and maintained at constant temperature in a water bath at 35°, 45° or 55°. Fruits (3–5) were removed at various times and a mesocarp powder prepared for immediate assay as described.

Preparation of extracts. For each extract, mesocarp slices were cut from the mid-region of several fruit (5–10) and the exocarp discarded. The slices were ground with liquid N_2 using a cooled mortar and pestle to form a fine frozen powder (mesocarp powder). For aq. extracts, 500 mg frozen powder was weighed and then a 1:5 (w:v) homogenate prepared in an ice-cold mortar with 2 ml, 200 mM Na-Pi buffer, pH 6.8, containing 0.5% Tween 20 with *ca* 500 mg acid washed sand (extraction medium). The homogenate was then centrifuged (8750 *g* max, 60 sec). Three layers were formed, (i) an upper lipid layer, (ii) a middle aq. layer containing small lipid globules and fat micelles and (iii) a lower layer of cell debris and sand. For NaCl extracts, concns of 0.25, 0.5 or 1.0 M NaCl were included in the extraction medium. Lipase activity under different pH conditions was determined either in 67 mM glycine–HCl, 67 mM NaOAc, 67 mM Na-Pi or 67 mM glycine–NaOH.

Requirement for lipid environment for lipase activity. Mesocarp slices (0.6 g) of fruit at different stages of development were added to the same weight of high-lipid containing mesocarp tissue (90A, 165 days after anthesis) in which lipase had been inactivated by heating the fruit for 10 hr at 45° (see Table 4b). A mixed mesocarp powder was then prepared as before by grinding in liquid N_2.

Lipase assay. An emulsion of glycerol tri-[1-^{14}C]oleate (55 μCi μmol^{-1}) in toluene (Amersham), was prepared fresh for each occasion. 27.3 nmol [^{14}C]trioleate (1.5 μCi; solvent removed with N_2 gas) was suspended in 100 μl EtOH and to this were added 100 μl 5% gum arabic (Sigma), 100 μl 5% CHAPS (Sigma), 100 μl 1% Tween 20 (BDH) and 200 μl of 200 mM Na-Pi buffer, pH 6.8, containing 5% bovine serum albumin (98–99% and essentially fatty acid and globulin free, Sigma) and 12 mM dithiothreitol (Calbiochem Novabiochem, U.K.). The mixture was vortexed for 5 min to produce a smooth emulsion. Cold triolein was not added to the cocktail as a substantial amount of non-labelled triglyceride is present in the mesocarp powder. The cold mesocarp powder was weighed very quickly (*ca* 10 mg) and placed in tubes pre-cooled with liquid N_2. 35 μl of radioactive cocktail (90–100 000 cpm) was then added to each tube. The tubes were vortexed and incubated for (usually) 30 min at the appropriate temp. Routinely, assays were carried out at a constant temp. between 16 and 18°. Each treatment was assayed in triplicate. For the aq. extracts 10 μl of the middle aq. micelle layer was assayed immediately following centrifugation and using the same cocktail mixture as for mesocarp powders. Speed was essential for the aq. layer would gel within 10 min if retained at 4°. In some experiments the upper lipid layer and the pellet were also assayed. All assay emulsions remained physically stable for at least 2 hr as judged by microscopic inspection at ×100 magnification. The reaction was stopped with 650 μl MeOH–$CHCl_3$–hexane (1.41:1.25:1.0). The [^{14}C]oleic acid released by lipase activity was converted into its K salt by adding 210 μl 50 mM K_2CO_3, pH 10, containing 50 mM K_3BO_3 and 50 mM KOH. The samples were vortexed and then centrifuged (Beckman Microfuge) for 60 sec. A sample (200 μl) of the upper aq. phase was then counted in 4 ml Cocktail T scintillant (BDH) in an LKB Wallac 1215 Rackbeta Liquid Scintillation Counter. Results presented are the mean ± s.d. value for a 200 μl sample from each triplicated assay. All solvents and chemicals were Analar grade, unless otherwise stated.

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Controlling fruit-shedding in the oil palm

Daphne J. Osborne, Janice Henderson and R. H. V. Corley

The oil palm is a major factor in the economy of much of South East Asia and maximising the harvest is of major consequence. One important source of loss is the shedding of ripe fruit from the bunches before they reach the factories. Study of the course of abscission reveals unusual processes in the oil palm – and in a few other plantation crops such as dates and coconuts – which suggest practical means of achieving better harvesting regimes and regulation of fruit shedding.

Oil palms (*Elaeis guineensis*) are now one of the world's most important plantation crops (figure 1) and in Malaysia palm oil is one of the country's major economic products. In parts of South East Asia, oil palms are estimated to outnumber the population by almost two to one. Whereas plantation management and the growing of oil palm, particularly of high-yielding material, has reached a high standard of efficiency, harvesting of the ripe fruit and its conveyance to the factory still involves losses that lead to unacceptable reductions in overall yield. The numbers of fruit produced by a palm (the promise of oil on the palm) is not satisfactorily reflected in the amount of oil recovered from the harvested bunches.

The cause of the shortfall is the non-synchronous ripening of the fruit on the different spikelets on any one bunch and the shedding and loss of the ripest fruit of the bunch before it reaches the factory. A recent and new understanding of the processes of ripening and shedding in the oil palm fruit offers a biotechnological approach to overcoming these losses. It combines the advances of clonal propagation programmes with the understanding of the unique physiological and biochemical controls that determine ripening and the timing of fruit-fall from the palm.

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The period of ripening

After fertilisation the fruit enlarges, reaching maximum size and fresh weight of both mesocarp and kernel by 120 days or so. Then, at a signal not yet understood in chemical terms, the cells of the mesocarp initiate a new range of gene expressions which include the synthesis of carotene and lipid and the production of at least two enzymes, a lipase and a cellulase. This is the start of ripening and for the next 30 or 40 days the intensity of the orange colour of the mesocarp increases as the carotene level rises; the commercially valuable palmitic, oleic and linoleic triglycerides in the flesh accumulate and the activity of the lipase reaches a maximum [1]. These are all concurrent events and they continue until another signal initiates a further set of gene expressions that herald the onset of fruit shedding. From the point of view of high quality lipid, the fruit should reach the processing plant just before, or at the time, that the shedding processes are initiated, for subsequently the lipase progressively functions in a hydrolytic role and the lipid levels start to fall. This results in the release from the triglycerides of the free fatty acids (again, mainly palmitic, oleic and linoleic) which, as they accumulate, increasingly spoil the quality of the oil extractable from the fruits. For the highest oil yield, though, harvesting should be delayed until a considerable number of the fruit have been shed, since lipid synthesis continues in the fruit that are still attached. At present, some loss of oil quality is accepted to maximise the yield, and where fallen fruit are collected, there is also a considerable additional cost. An alternative solution to the problem, therefore, is the elimination of the shedding process altogether. Current research suggests that suitable genetic manipulation of the oil palm could offer this possibility.

The process of shedding

The anatomy of abscission of the oil palm differs from that of most commercial fruits. Instead of a synchronous series of cell separations across a plane of cells between the fruit and the stalk, resulting in immediate shedding of the fruit above, the oil palm undergoes abscission in two distinct stages with a time lag of 1–2 days between the two [2].

The flower of the oil palm is hermaphrodite, but normally only one of the sexes will complete development in any one bunch so that sequential bunches will carry only male or female spikelets. In the female, the staminal ring aborts before anthesis and is left as a circlet of tissue, the rudimentary androecium, surrounding the base of the ovary and immediately adjacent to the inner whorl of papery tepals (figure 2a, b). (Tepals are elements in the perianth of a flower lacking differentiated sepals and petals.)

As the fertilised fruit enlarges, the cells of this rudimentary androecium and the tepal bases continue to divide, keeping pace with the increase in diameter of the base of the fruit. This pattern of differentiation results in the fruit being attached to the spikelet at a junction with three distinct tissue types. The first (position 1) is the base of the fruit itself (figure 3a, c) and here, even before anthesis, the sites of cell separation are clearly defined by a line of small cells with dense cytoplasmic contents. This is the destined site of the first stage of the abscission process and separation at this position occurs only when the fruit is fully ripe. Even when cell separation at position 1 is complete, the fruit is not shed, for the cells of the circlet of the rudimentary androecium and the bases of the tepals still adhere closely to one another (positions 2 and 3, figure 3b and c). The fruit is, however, loosened and at this stage can, in the wild, be readily plucked from the bunch by primates, parrots or other fruit feeders.

The second stage of abscission, which

Figure 1 A young elite oil palm with developing fruit bunches.

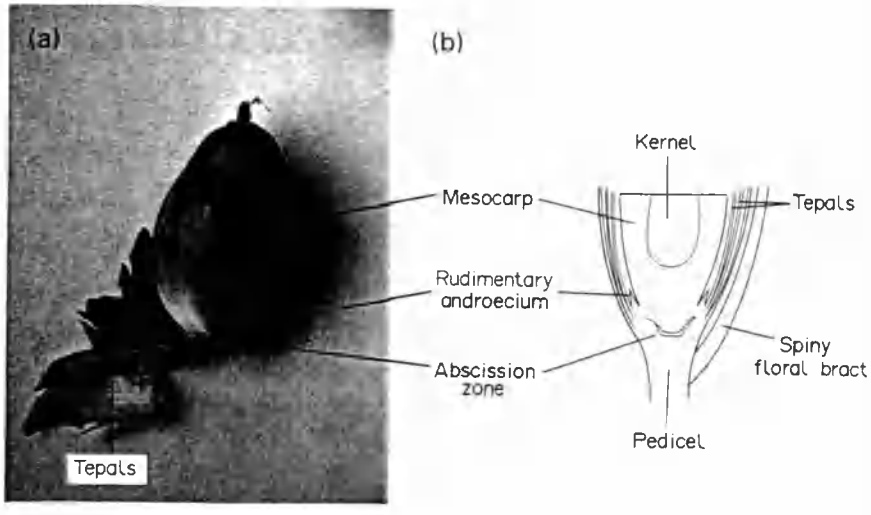


Figure 2 (a) Mature fruit showing the stamen primordia (rudimentary androecium) the tepals and the mesocarp. (b) Diagrammatic representation of the longitudinal section of a mature fruit.

esults in the fall of the fruit to the ground, ccurs only after the first stage is complete nd involves a special role for the cells of he rudimentary androecium. Usually, the ells of the circlet immediately adjacent to he fruit base (position 2), though frequently some of those adjacent to the tepal ases (position 3), will undergo separation figure 3c) so that the fruit falls free from he enclosing tepals. The upper parts of the epals are by then brown and dry. The shed ruits are therefore either naked of any asal attachments (figure 4a) or may have ragments of the rudimentary androecium till adhering.

A naked fruit leaves a complete circlet f its rudimentary androecium still attached to the tepal bases and partly to the pad of stalk tissue at position 1 (figure 4b), but where some of the androecial ring clings to the fruit, the remaining tissue of the circlet stays with the tepals on the spike.

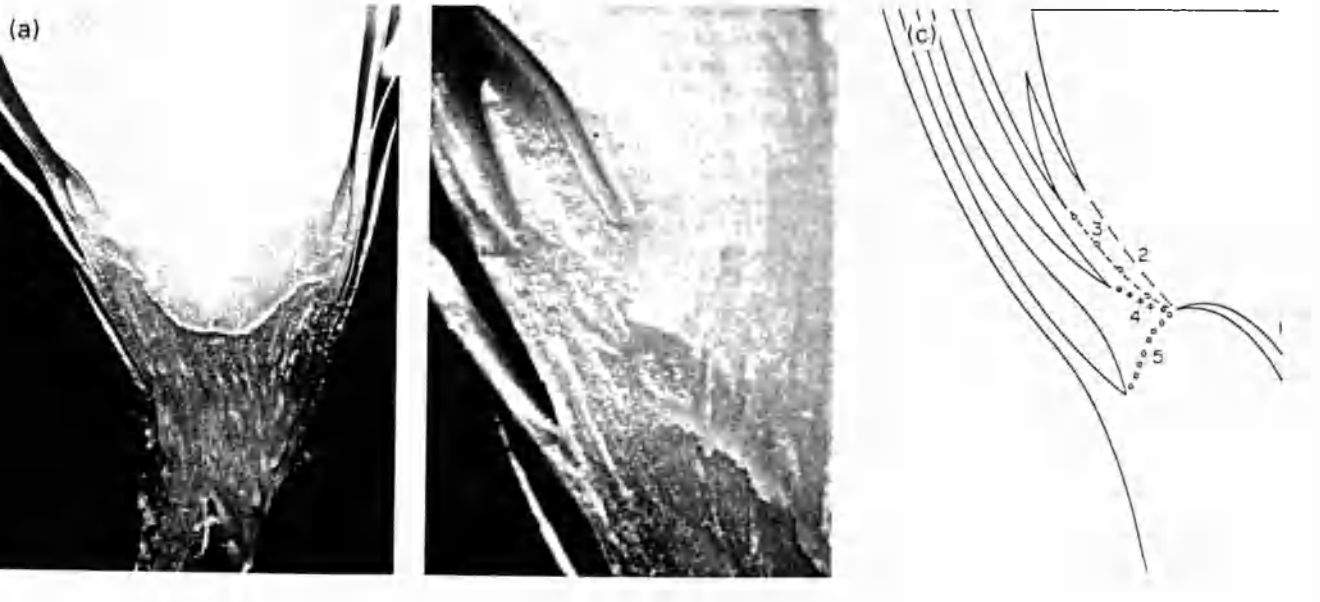
In exceptional circumstances, when spikes of fruit have been removed from a bunch before they are fully ripe, fruit shedding will eventually occur across the bases of the tepals (positions 4 and 5). Even in these conditions, separation always occurs first at position 1, to be followed by the second stage at either position 4 or 5. Such fruit are therefore shed still enclosed in the ring of tepals (figure 4c) and normal separation at positions 2 and 3 at the margins of the rudimentary androecium is bypassed.

The signal for harvest

On the plantations, palms are checked every few days for bunches at the appropriate stage of ripeness for cutting. Conventionally, the falling of a few ripe fruit to the ground is taken as the harvest signal, and for the taller palms this affords proof of ripeness of a bunch that is otherwise difficult to see among the bases of the fronds. But before the bunch reaches the factory many more fruit are shed and so a yield loss is established

A sexual aberration

Long ago, occasional abnormal fruit development was reported to occur in certain oil palms [3]. In these, the rudimentary androecium did not remain as a circlet of tissue but instead enlarged and developed



igure 3 Longitudinal sections of mature fruit showing (a) cell separation (abscission) at the base of the fruit (position 1) and (b) ose-up of the sites of final separation at the rudimentary androecium or tepal bases (positions 2 and 3). (c) Diagrammatic epresentation of the sequence of potential positions for cell separation.

igure 4 View of fruit shed naked (a) and (b) showing the complete androecial ring remaining within the tepals on the spike. (c) ruit shed with all tepals attached.

to six (usually) additional seedless lobes f female mesocarp (supplementary arpels) surrounding the central fruit (fig- e 5a, b). These parthenocarpic lobes syn- esised carotene and lipid and ripened in oncert with the kernel-containing fertile vary. This additional lipid-rich mesocarp ffered a potential for high yields, and cer- in seedlings and at least one genetic line oil palm was found which routinely pro- uced such fruit. The promise of high elds from these so-called 'mantled' fruit as not fulfilled, however, perhaps be- use, although the fruit ripened, it was not ed. In the absence of the usual signal of e first few ripe fruits that fall to the round, bunches on the mantled palms ere left unheeded and the fruit were ick to rot on their spikelets.

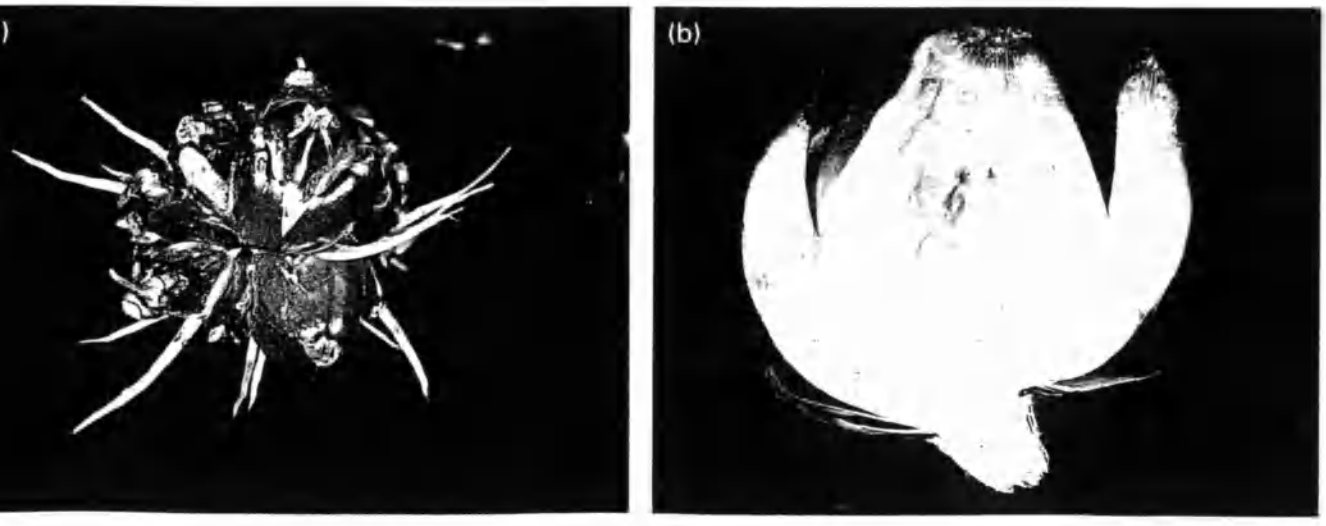
lonal oil palms

the 1980s, great efforts were made to grade the yields of lipid by the introduc- on of clonal plant material raised by sue culture from root or shoot fragments ken from elite, high quality, high lipid- oducing palms. Many thousands of these onally propagated individuals are now aring fruit in plantation trials around the orld and improved yields have resulted om these plantings. Certain of the tissue culture procedures, involving the use of plant hormones in the media have, however, also led to a proportion of the palms showing sexual abnormalities that resemble the naturally occurring mantled fruit [4]. While the rudimentary androecium may form very well-developed lobes of supplementary carpels that extend the whole circlet of the androecial ring, sometimes, only one or two small lobes may arise while the remainder of the ring may be normal. In such fruit, abscission occurs normally at position 1, but at positions 2 and 3, cell separation takes place only where the rudimentary androecial ring has remained as aborted staminal tissue. Where the ring has differentiated into mesocarp tissue, the fruit remains attached to the bases of the tepals (figure 6a, b).

Control of this second stage of fruit abscission, and hence of fruit shedding, can therefore be manipulated by altering the developmental programme of the cells of the rudimentary androecium early in differentiation and before anthesis. Evidence from clonal propagation biotechnology now indicates that the levels of hormones used in tissue culture can determine the degree of mantling expressed by a palm several years later when it starts to flower. Because the condition does not show constant expression it may have an epigenetic origin within these highly specialised cells, but RFLP (restriction fragment length polymorphism) analysis for certain of the clones expressing mantling indicate that DNA genomic changes may also have occurred between the parent palm and the tissue culture progeny [5]. Although the mechanism by which mantling is directed remains unresolved, the techniques by which it can be induced are now known, affording new possibilities for manipulating abscission.

Inter-tissue signalling

In those crops where fruit shedding has been closely studied, the signal for abscission can be directly linked to critical levels of ethylene produced by the ripening fruit and perceived by the ethylene-responsive target cells of the abscission zone. In apples, oranges or tomatoes, for example, the synthesis of ethylene increases as ripening proceeds, and at full ripeness the levels reach a threshold that initiates in the zone cells an expression of new cell wall modifying enzymes (glucanhydrolases) that loosen adhesion at the zone cell interface with neighbour cells. Because the line of separation is so precise (despite the fact that the secreted enzymes may migrate



gure 5 (a) Spikelet of ripe genetically mantled fruit. (b) Longitudinal section of a mantled fruit.

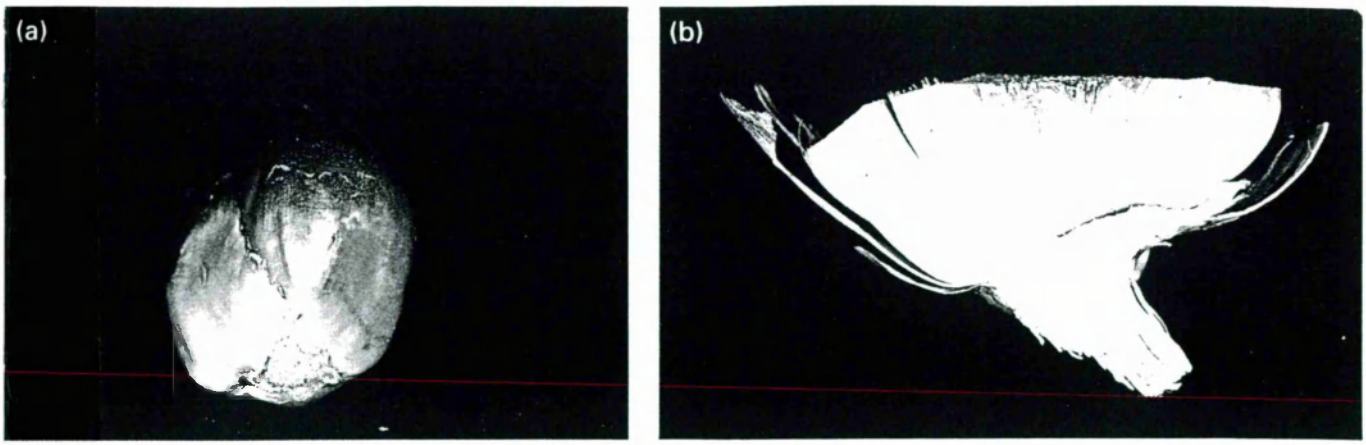


Figure 6 (a) A clonal, partially mantled ripe fruit with one supplementary carpel only (LHS) (b) Longitudinal section showing separation at the fruit base (position 1) and site of eventual separation at position 2 only where the rudimentary androecium is present (RHS). No abscission at the position of the supplementary carpel (LHS).

considerable distances through the cell walls of adjoining tissues), it is evident that substrate specificity exists between the secreted enzymes and the walls of a limited number of cells which are restricted to the immediate vicinity of the zone [6]. In this way, only certain cells become separated from their neighbours by the enzymes that are induced in the zone. In these dicotyledonous fruits, once the abscission cascade of enzymes is produced (diagnostically, this usually includes a specific 9.5 pI isozyme of β-1, 4-glucanhydrolase) separation is initiated across all the cells of the fruit stalk and the fruit is shed. In the oil palm, the situation is different. Throughout ripening, during the rise in lipid and carotene, the levels of ethylene produced remain insignificant. It is not until the fruit is deep orange in colour and near maximum lipid content that ethylene synthesis starts to rise. This occurs first at the apical end of the fruit and quickly progresses towards the base. In spikelets that are removed from the bunches, ethylene production by a fruit reaches a maximum

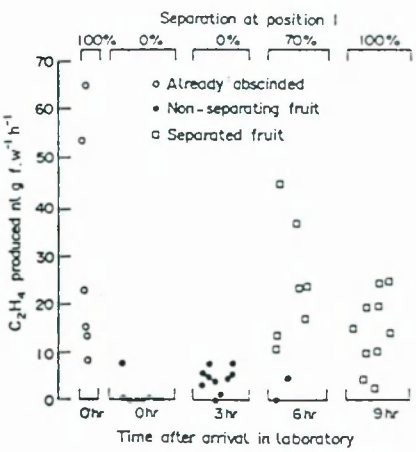


Figure 7 Rates of ethylene production by single fruits in relation to the onset of separation (abscission) at position 1. Fruit spikes harvested from the Malaysian plantation 24 hr before sampling and analysis in the U.K.

within 24 hr of the initial rise and thereafter declines. Ethylene production and the onset of cell separation at position 1 indicate that these events are very closely linked. Time-course experiments have shown that only those fruit that exhibit the rise in ethylene synthesis initiate separation at the fruit-pedicel junction (position 1) figure 7.

It is not mesocarp tissue, however, that abuts directly onto zone cells. Instead, several layers of cells that synthesise neither carotene nor storage lipid provide a narrow barrier between the mesocarp and the cells of the zone. It is at the junction of these 'barrier' cells and the zone cells that the first wall separations take place. The second stage of separation at positions 2 or 3 appears to be dependent upon the achievement of the first stage of separation at position 1, and not directly upon the signal of ethylene. Another signal, generated by separating cells at position 1 (and possibly an oligosaccharide) would appear to pass to, and be perceived by, the cells that constitute positions 2 and 3. During this second stage of abscission, cells of the rudimentary androecium dissociate from each other and from other cells adjacent to them. Since these other cells include those at the outer edge of the base of the fruit, the pedicel and the bases of the tepals, several distinct cell types are intimately involved. When the rudimentary androecium develops as mesocarp, as in the formation of the mantled condition, cells are differentiated that no longer have the competence to participate in cell separation responses to abscission-inducing signals, and the intimate complex of inter-tissue signalling fails.

An orchestra of enzymes

In dicotyledonous fruits and leaves, abscission is linked to the induced expression of the 9.5 pI isozyme of β-1, 4-glucanhydrolase (cellulase) by the separating cells of the zone. This is not so in the oil palm fruit. Although ripening mesocarp tissue produces a cellulase, the zone does not. Furthermore, the cells of the mesocarp do not separate despite the production of cellulase isozymes. Instead, at position 1 of the oil palm fruit, an active polygalacturonase is produced at abscission, indicating break-down of uronide linkages in the zone region, while at positions 2 and 3 high levels of a β-1, 3-glucanhydrolase are induced. Although these enzyme activities may in part reflect the overall differences between the cell wall composition of dicotyledons and monocotyledons, they also serve to illustrate the differences between the major enzymes induced in the two distinct stages of abscission in the oil palm fruit [7].

Potential solutions to the problem of fruit shedding

A number of ways are now open for the genetic manipulation of the oil palm: these should reduce the losses that result from unwanted shedding. Each could be engineered during the clonal stages of propagation in tissue culture.

Firstly, the production of ethylene could be blocked in the fully ripe fruit by the incorporation of antisense genes to the important enzyme ACC-synthase. This enzyme controls the synthesis of the ethylene precursor ACC. Antisense mRNA production has already been achieved for the tomato, thereby preventing translation of the normally produced sense mRNA and inhibiting the rise in ethylene formation by mature fruit, so delaying ripening [8]. It would be important, however, that the antisense gene be expressed only in the ripe fruit and not in all other cells of the palm, for enough is not yet known about ethylene control of other aspects of palm development. This control may be achieved through the use of gene promoters specific to fruit tissue. Alternatively anti-sensing ethylene-response genes may give greater specificity. In this case a promoter specific to abscission zone cells would be required.

Secondly antisense could be introduced for the polygalacturonase gene of position 1 or for the β-1, 3-glucanhydrolase gene of

ositions 2 and 3, again under the control f the appropriate promoters. Since it is kely that specific isozymes of the enymes are synthesised only at these separaon positions, such options offer a precise evelopmental control of gene expression iat is restricted to the abscission zone one.

Thirdly, biotechnology could be further dvanced so that the tissue cultures from hich elite palms are propagated could be o precisely manipulated hormonally that ie rudimentary androecium would consisntly develop on the female inflorescences s a narrow circlet of mesocarp cells. This ifferentiation would then permit fruit loosning at position 1, but preclude the possiility of the second stage of the fruit oscission process at positions 2 or 3.

Whenever any one of these options beomes a reality – and there is no doubt that ach would increase the total harvestable ield of fruit – they will raise a serious practical problem for the men who work on the plantation. If the fruit are no longer shed, so that none falls upon the ground, how will the cutters know exactly when each bunch has fruit that are fully ripe and so be able to judge exactly when each bunch should be harvested? To resolve this, new practices for monitoring ripeness in the field must now be developed.

Overall, the importance of these researches is the promise they now offer for operating more regulated fruit shedding and harvesting regimes not only for the oil palm, but for other major palm plantation crops of high value including dates and coconuts.

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Inter-tissue signalling during the two-phase abscission in oil palm fruit

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Abstract

Shedding of oil palm fruit from the spike takes place in two stages. The first, cell separation at the junction of the fruit base and the pedicel (position 1) is initiated by ethylene or its precursor (ACC) or by treatments that accelerate the production of ethylene (ABA). Separation is delayed or suppressed by treatments that block ethylene biosynthesis (AOA) or oppose ethylene action (auxin, 2,4-D). Separation of cells at the fruit base from the rudimentary androecium or from the ring of tepals at the pedicel edge is a second stage event that depends upon the achievement of separation at position 1. Abscission cells differentiated at these secondary positions do not separate in response to ethylene or to ethylene enhancing compounds alone. It is concluded that a chemical stimulus from the separated position 1 provides the signal that induces the second cell separation process essential to the completion of fruit shedding.

Key words: Oil palm, *Elaeis guineensis*, fruit abscission, ethylene, cell separation, inter-tissue signalling.

Introduction

The non-synchronous shedding of oil palm fruit as each individual fruit ripens raises a problem in timing the harvesting of these many-fruited bunches in the field. The lipid content of the fruit reaches its maximum at the time of, or possibly just after, shedding so the control of fruit-fall is critical to obtaining the highest overall yield (Chan *et al.*, 1972).

In an earlier study we showed how shedding is a two-phase process. First, the fruit separates from the central region of the pedicel (position 1, Fig. 1) but remains adhering to the fruit stalk by a circlet of non-separated tissue which includes the base of each of the enclosing six tepals and the continuous ring of the rudimentary androecium (Henderson and Osborne, 1990). In the field, cells at the secondary sites of positions 2 and/or 3 then undergo separation and only then is the ripe fruit free to fall from the bunch. This second phase follows upon the fruit-pedicel (position 1) separation after an interval of 1 or 2 d.

The terminal fruit on each spikelet of a bunch shed first, for these are the fruit that ripen first. Since the shed fruit are not always collected, they can be a major cause of yield reduction. Field experiments with growth regulators have so far shown variable responses of enhancement and repression of fruit fall in the oil palm (Chan *et al.*, 1972). Although ethylene can initiate fruit and leaf shedding in dicotyledons (Reid, 1985; McGlasson, 1985;

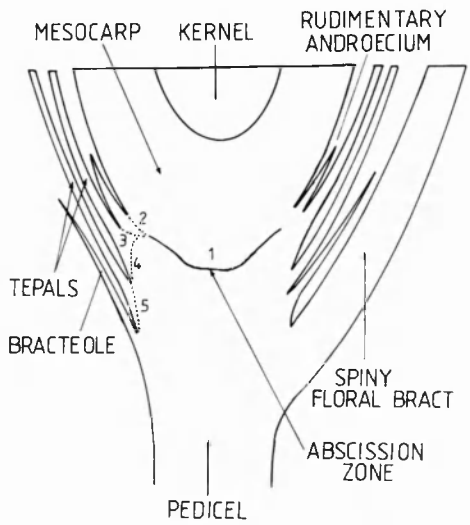

Fig. 1. Diagrammatic representation of a longitudinal section across the abscission region in the oil palm fruit indicating the sequence and position of cell separation events between the different tissue parts. Nomenclature for the different floral parts is based on that of Van Heel *et al.* (1987).

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Osborne, 1989; Goren, 1993; and references therein) and promotes perianth abscission in monocotyledons (McKenzie and Lovell, 1992) alternative regulatory mechanisms that are independent of ethylene exist. In a number of species in the Gramineae, for example, the mature fruits are shed by an active separation process that is initiated by abscisic acid but is unresponsive to ethylene (Sargent *et al.*, 1984). There is the possibility, therefore, that other monocotyledons, including the palms, might exhibit novel controls in ripening and abscission.

In order to elucidate the signalling events that initiate and complete the two-phase shedding of oil palm fruits and to follow the biochemical changes that lead to cell–cell dissociation in the different target tissues of the separation zone, we have re-investigated the role of ethylene and other hormonal regulators in the abscission process.

We now report on the complex interactions between the ripening fruit and its enclosing appendages that contribute to controlling the timing of abscission in *Elaeis guineensis*.

Materials and methods

Plant material

Freshly-harvested spikelets of clonal material (90A, 926, 271D, and 476G) were shipped air-freight from Malaysia in loose cotton bags enclosed in perforated polythene and boxed in a perforated cardboard container. At no time during transit did the temperature of the fruits fall below 12 °C nor did it rise above that experienced by fruit in the field (maximum 40 °C). Spikelets were received in Oxford within 24–30 h of excision from the parent palm and were used as the experimental material. Each spikelet was a known number of days from anthesis and consisted of 10–40 fruits.

Seasonal differences in rates of fruit maturation do not permit direct comparisons of data at the same days after anthesis between the different tables and graphs but comparisons within each set of data are valid. Each experiment has been conducted at least three times and the data presented (with the replicates) are for one of these experiments. The relative differences between treatments were similar for each of the experiments carried out.

Assessment of abscission

On the plantations, ripe fruit are normally shed naked or with small patches only of rudimentary androecium adhering. This indicates the critical participation of cells at position 1 and, additionally, by cells at the base of the rudimentary androecium (positions 2 or 3) during the abscission process, (Fig. 1). After the fruit is shed, the tepals remain attached to the spike. In excised spikelets that either shed their fruit in transit to UK (or start separation at position 1 during this transit period and then shed), the remaining tepals will separate cleanly from the pedicel (positions 4 and 5) within 48 h if the spikelet is maintained at a >75% relative humidity.

To determine if separation of the fruit had taken place at position 1 in fruit that was still attached to the spike, fruit were cut in half longitudinally with a Stanley knife and gentle pressure exerted on the fruit side and on the pedicel side of the zone. Non-abscinding fruit will not separate under these circumstances, even when great force is applied, but fruit that is partly separated, at position 1 readily dissociates one side from the other and this is easily seen by eye. During this first stage of fruit abscission, the tepal bases and the rudimentary androecium remain attached to the fruit and pedicel.

The complete separation of the fruit from the tepal bases and rudimentary androecium (position 2, or 2 and 3) permits the naked (or almost naked) fruit to be plucked from the spike without force, leaving the tepals and all (or most) of the rudimentary androecium still attached to the spike.

Fruit that is not fully ripe on arrival in the UK will, after several days, complete the first stage of separation at position 1, but will, however, bypass the normal second phase of separation at positions 2 or 3, and instead separate at positions 4 and 5 (Fig. 1). In these circumstances, the fruit is shed with all tepals attached. Then, only the large spiny bract and the two small bracteoles are left upon the spikelet.

Measurement of ethylene by gas chromatography

Ethylene analysis was by gas-solid chromatography on a Pye-Unicam series 104 gas chromatograph fitted with a flame ionization detector and hydrogen and air ignition gases. An alumina F1 column with nitrogen carrier gas was used for separation with analysis temperature and flow rates as described by Ward *et al.* (1978). Whole spikelets were enclosed in 1.5 l Kilner jars with Suba-Seals inserted into the lids; individual fruit or fruit parts were held in 28 ml glass vials, also sealed with a Suba-Seal. For each determination, 1 ml of the gas phase within each container was drawn through the Suba-Seal with a gas-tight hypodermic syringe. Results are expressed as nanolitres ethylene produced per gram fresh weight per hour.

Ethylene production studies

Ethylene production by shed or separating fruit was always high and was therefore measured after a series of *c.* 15–20 min incubations. Longer incubations (1 h or more) were necessary to detect production in ripening or unripe fruit. Production was monitored from the time of arrival in the laboratory and thereafter at various intervals up to 3–4 d. During the aeration periods between measurements, containers were covered with Parafilm.

(a) *Whole spikelets*: Immediately upon arrival in the laboratory, spikelets were weighed and placed in the Kilner jars and sealed. The ethylene production was measured after periods of incubation (*c.* up to 1 h) at 24 °C and thereafter at various intervals for the following 40 h.

(b) *Individual non-separated fruit*: Individual fruit with their attendant tepals were stripped from the spikelet and the fibres of the pedicel trimmed to 5 mm below the abscission zone. The tepals were either completely removed to give a naked fruit with only the rudimentary androecium remaining attached, or the fruit was used with all tepals still intact.

(c) *Individual separating fruit*: These fruit, in which only position 1 had already separated, were gently removed from the spikes so leaving the tepals and rudimentary androecium attached to the pedicel within the spikelet.

(d) *Individual shed fruit*: Shed fruit were those that were fully ripe but still attached at the time of harvesting in the field. Separation at position 1 was almost certainly in progress at

harvest, but the second phase separation at positions 2 or 3 would have occurred during the flight to the UK. These fruit were, therefore, loose in the cotton bag on arrival 24–30 h after the spikelets were harvested. They were shed naked with no adhering tepals and traces only of the rudimentary androecium still attached to the fruit base.

(e) *Individual parts of the fruit and spikelet*: Tepals, mesocarp slices, rudimentary androecial tissue and excised abscission zones from unseparated fruit as well as the loosened abscission zone cells from abscinded fruit, were all assayed for ethylene production at different stages of development from anthesis up to fruit shedding. The methods for ethylene collection were the same as for individual fruits.

(f) *Wound ethylene*: All parts of the fruit were assessed for the extent of wound ethylene production by 15 min determinations over a period of 2–3 h. All parts exhibited an increase in the production of ethylene after 30 min (except the mesocarp of ripe fruit) so the duration of the sampling period was kept to a minimum. The ripe mesocarp showed no rise over a period of 2 h from cutting.

Other growth regulator studies

A series of experiments were undertaken, using different growth regulators, to follow ethylene production, and the regulation of abscission in the different abscission-associated tissues of ripe, ripening and unripe fruit.

(a) Treatments with 1-aminocyclopropane-1-carboxylic acid (ACC), 2,4-dichlorophenoxyacetic acid (2,4-D), abscisic acid (ABA), and 1-amino-oxyacetic acid (AOA) (all from the Sigma Chemical Co, Ltd) were assessed over a range of concentrations from 10 μM to 1 mM. For this, solutions of the sodium salts in distilled water were adjusted to pH 6.8–7.0.

Controls were treated with distilled water.

(i) For ethylene production or abscission measurements on whole fruit, single fruit with the fibres of the pedicel trimmed to 5 mm from the abscission zone, were placed in 500 μl of the treatment solution in a 28 ml vial. This was sufficient to submerge the ends of the pedicel fibres but left the abscission zone region unwetted.

(ii) For excised fruit parts (tepals, rudimentary androecium, mesocarp slices) tissue (0.5–1.5 g) was enclosed in the 28 ml vials with the addition of 500 μl of the treatment solution.

(iii) For treatments of the tepals attached to the pedicel, but with fruit and rudimentary androecium removed, a 1 cm length of the pedicel fibres was inserted into 1.0 ml solutions in Eppendorf tubes so that the tepal bases were held above solution level.

(b) For direct exposure to ethylene, whole spikelets or isolated fruit or fruit parts were enclosed in 12 l tanks. Ethylene gas (diluted to 5000 μl l^{-1} in air) was injected through a Suba-Seal port to give the desired final ethylene concentration (usually 5–10 μl l^{-1}). For controls, dishes containing *c.* 20 ml of mercuric perchlorate (MP) 0.25 M in 2.5 M $HClO_4$, were enclosed with the fruits or fruit parts to absorb ethylene liberated by the plant tissues. Containers were aerated regularly at not more than 24 h intervals, re-sealed and re-injected with ethylene.

(c) For treatments of tissue slices, fruit of different numbers of days from anthesis were stripped from the spikelets and severed horizontally across the mesocarp below the kernel leaving the base of the fruit and the abscission zones of the rudimentary androecium and the tepals still intact. Two median longitudinal slices were then cut from each fruit to provide *c.* 2 mm thick sections that included the abscission zones at positions 1, 2, 3, 4, and 5. Sections (usually 10) were placed (but not immersed) in a shallow layer of the solution under test and assessed (by gentle pressure) for separations at the different abscission sites after 24 or 48 h. Other fruit, with all tepals removed, were similarly treated to provide slices with all of position 1 present. Chloramphenicol at 10 μg ml^{-1} was included in the medium to suppress growth of microorganisms.

Results

Experiments with growth regulators

Fruit shedding from spikelets: To assess the direct effect of ethylene and an auxin (2,4-D) on the shedding of fruits from whole spikelets, samples of increasing numbers of days from anthesis were either (i) enclosed in 12 l tanks with open dishes of MP, (ii) enclosed in 12 l tanks into which ethylene was injected to a concentration of 10 μl l^{-1}, (iii) immersed into beakers containing distilled water or a solution of 0.5 mM 2,4-D with 0.002% Tween 20 for 1 h, then air-dried, enclosed in cotton bags and each treatment held in a 12 l tank in air.

The series of graphs (Fig. 2) show that ethylene enhances and 2,4-D delays the shedding of fruit from the spikelets. Fruit were shed with all tepals attached, separating at positions 1, 4 and 5. The enhancement and delay is greatest in the younger unripe or ripening fruit. Such results indicated that the normal dicotyledon-type abscis-

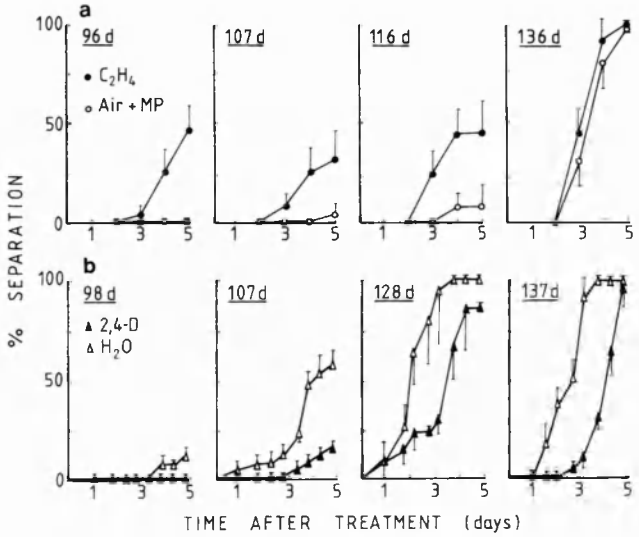

Fig. 2. Acceleration and retardation of shedding of fruits from spikelets of different numbers of days post-anthesis. Spikelets (3) treated with (a) ethylene, 10 μl l^{-1} or (b) 2,4-D, 0.5 mM. For details see methods. For each treatment $n = >30$. S.E. bars = observed % abscission at each time (binomial distribution).

sion regulation by auxin and ethylene was likely to operate, but that in fruit approaching full ripeness additional controls must determine the final shedding events.

Shedding of individual fruit with tepals intact: For these, individual fruits were stripped from the spikelets to give fruits with all six tepals and the rudimentary androecium still attached and with the pedicel fibres trimmed to 5 mm in length. Fruits of increasing numbers of days from anthesis were enclosed in 12 l tanks and exposed to either ethylene ($10\ \mu l\ l^{-1}$) or to air with dishes of MP to absorb ethylene. Ethylene enhanced shedding, particularly in the younger fruit (Table 1). All fruit were shed with the two whorls of tepals still attached, i.e. separation taking place at positions 1, 4 and 5.

Shedding of fruits with tepals removed: Experiments with fruit from which all tepals were removed before exposure to ethylene or to air + MP (Table 2) confirm that separation at position 1 is enhanced by ethylene and retarded if ethylene is removed from the ambient air by the presence of MP.

Shedding of attached tepals only: Individual fruits were stripped from unripe spikelets (up to 120 d from anthesis) and cut to remove the fruit tissue leaving only some of the rudimentary androecium, and all six tepals attached to the abscission pad of the pedicel stalk. These de-fruited stumps with a 1 cm length of the pedicel fibres below were then exposed to ethylene ($10\ \mu l\ l^{-1}$) or to air + MP as described before, and recorded over 3 d. Although this material was maintained at >75% relative humidity, no shedding occurred in the air controls and no shedding of the tepals could be induced by ethylene despite the presence of separation positions 3, 4 and 5. Both tepals and the remains of the rudimentary androecium remained firmly attached to the pedicel.

In other experiments, ripening fruits (133 d from anthesis) were cut in the same way as before to leave six tepals attached to the abscission pad of the pedicel stalk. The pedicel fibres were then inserted into either 1 ml water or solutions of ACC or ABA up to 1 mM as described in methods. No separation of tepals occurred in any of the treatments during the experiment (4 d) indicating that these ethylene-promoting growth regulators do not directly initiate abscission at these potential abscission sites.

Lastly, spikelets in which the ripe fruits were shed during transit to UK (leaving the six tepals attached to the pedicel stalk) were either enclosed on arrival within containers of air or air with $10\ \mu l\ l^{-1}$ ethylene, or the bases of the spikelets were inserted into beakers of distilled water or into a solution of ACC 1 mM. Under these conditions all the tepals loosen from the pedicel by 48 h, but no acceleration of separation occurs in the spikelets receiving ethylene or ACC.

Together, these results suggest that only the fruit-base/pedicel separation zone (position 1) is under a direct regulation by ethylene. The subsequent separations at positions 2 and 3, or 4 and 5 are apparently not directly responsive to ethylene, but instead could be/are subject to other controls; these could include signal substances liberated by the separating cells at position 1.

Table 1. *Percentage shedding of individual isolated whole fruit (tepals and rudimentary androecium attached) 4 d after enclosure in either ethylene $10\ \mu l\ l^{-1}$ or in air + MP*

Days from anthesis	% Shedding[a]	
	C_2H_4	Air + MP
120 ($n=13$)	69	0
133 ($n=16$)	63	0
162 ($n=19$)	63	11

[a] All fruit were shed with their tepals and rudimentary androecium still attached, i.e. separation at positions 1, 4 and 5.

Table 2. *Percentage abscission at position 1 of individual isolated whole fruits (with tepals removed) after exposure to ethylene $10\ \mu l\ l^{-1}$ or air + MP for 4 d*

Days from anthesis	% Abscission	
	C_2H_4	Air + MP
121 ($n=17$)	41	0
133 ($n=6$)	33	0
155 ($n=15$)	87	27

Confirmation of ethylene control of position 1 abscission

(a) *Whole fruits with tepals removed*: Whole fruits, 118 to 148 d from anthesis and with intact abscission zones were stripped from the spikelets, all the tepals and outer appendages were removed and the fruit placed in tanks with either $5\ \mu l\ l^{-1}$ ethylene, or in air + MP. At intervals of time from the start of the experiment fruit were sampled and cut open to determine if cell separation had been initiated at position 1. The results show (Fig. 3)

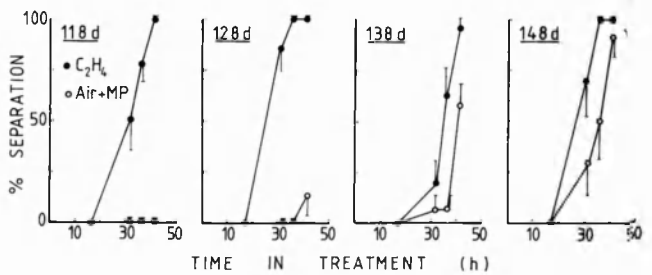

Fig. 3. Acceleration of cell separation at position 1 in ripe (148 d), ripening (138, 128 d) and unripe (118 d) individual fruit (tepals and outer appendages removed) exposed to ethylene $5\ \mu l\ l^{-1}$ compared with fruit maintained with MP to deplete ambient ethylene. For each treatment, $n=>30$. S.E. bars = observed % abscission at each time (binomial distribution).

(a) *Whole fruits with tepals removed*: Whole fruits, 118 to 148 d from anthesis and with intact abscission zones were stripped from the spikelets, all the tepals and outer appendages were removed and the fruit placed in tanks with either $5\ \mu l\ l^{-1}$ ethylene, or in air + MP. At intervals of time from the start of the experiment fruit were sampled and cut open to determine if cell separation had been initiated at position 1. The results show (Fig. 3)

that cell separation at this site occurs earlier in the fruit exposed to ethylene than in those fruit from which ethylene was depleted from the ambient air by MP. Also, the acceleration due to added ethylene is less in fruit nearing full ripeness.

(b) *Tissue slices from fruits with tepals removed*: For these experiments, the tepals were removed from the fruits before the tissue slices were cut as described in methods.

After 24 h in contact with solutions of 2,4-D, ABA, ACC, water, or with water and exposed to air or ethylene 5 $\mu l\,l^{-1}$ it was evident that ABA, ACC or ethylene all enhanced the rate of separation across position 1 whilst 2,4-D retarded the process and curtailed the enhancement due to ACC. (Table 3).

(c) *Tissue slices from fruits with tepals attached*: Tissue slices cut from fruits with tepals attached and exposed in a similar way to treatments described in (b) showed acceleration of separation of all positions with ethylene, ABA or ACC and retardation with 2,4-D. Results indicate that separation at positions 4 and 5 can occur if the separation positions 1, 2 and 3 are also present and separating.

Treatments with ethylene, or with the ethylene precursor ACC (Adams and Yang, 1979) or with ABA that promotes a rise in ethylene production (Table 8) can therefore induce a premature cell separation at position 1, whilst auxin (2,4-D) treatments can oppose this separation.

Ethylene production by individual fruit parts

Since it was evident that abscission at position 1 could be regulated by ethylene or by growth regulators that enhance ethylene production, it was necessary to determine which of the tissues neighbouring the zone might act as the source of an ethylene signal.

Table 3. *Enhancement and retardation of abscission at position 1 in longitudinal tissue slices of 148 d post-anthesis fruits containing this potential position for cell separation*

Results recorded 24 h after immersion in solutions of the growth regulators. Each treatment contained 11 fruit slices. Lower concentrations (0.25 and 0.1 mM) gave lower responses and are not included.

Treatment	% Abscission		
	Fully separated	Partly separated	Intact
H_2O	0	0	100
ACC (0.5 mM)	27	18	55
2,4-D(0.5 mM)	0	0	100
ACC (0.5 mM)+ 2,4-D (0.5 mM)	9	0	91
ABA (0.5 mM)	45	18	37
ABA (1.0 mM)	82	0	18
Ethylene(5 $\mu l\,l^{-1}$)	47	21	32

(a) *Whole spikelets*: The production, on arrival, of intact spikelets enclosed in 1.5 l Kilner jars showed no appreciable level of ethylene emission by unripe (105 d) or ripening material (124 days post-anthesis). Spikelets (140 d) with ripe fruit at the just pre-shedding (position 1 separating), or shedding stage (157 d post-anthesis, position 1 separated) showed high production levels (Table 4).

(b) *Intact fruits*: Assessment of ethylene produced by fruits at different stages of ripeness (days from anthesis) showed levels that accounted for almost all of the production of the whole spikelet (Table 5), with the highest levels in fruits at a late pre-shedding stage (151 d, position 1 still intact).

(c) *Individual fruit parts*: Dissection of the fruits into tepals, rudimentary androecium, mesocarp, and abscission zone showed that the major source of ethylene production (nl g^{-1} Fwt of tissue) is the mesocarp of ripe separating fruit. On a per fruit basis, the tepals and the rudimentary androecium make only a small contribution at this stage (Table 6).

(d) *Positional production of ethylene in ripe fruit mesocarp*: Analysis of ethylene production by different parts of the fruit mesocarp shows that the rise in ethylene synthesis starts first at the apical (stigma) part of ripe fruit and progresses basipetally, towards the abscission zone. Separation at position 1 in these different samples of ripe fruit, coincides with the high level of production of the

Table 4. *Ethylene production levels by whole spikelets, each consisting of 7 or 8 fruit: values are means with range of duplicates from one experiment*

Days from anthesis	nl g^{-1} Fwt h^{-1}	
	0–1 h	3–4 h
105*	0.9±0.1	0.3±0.1
124*	2.1±0.4	1.0±0.4
140**	51.6±8.4	86.7±15.9
157***	57.1±2.7	62.4±12.9

* Spikelets with non-separating fruits.
** Spikelets containing fruits just separating at position 1.
*** Spikelets with fruits separated at position 1.

Table 5. *Ethylene production by individual intact fruit at different stages of ripeness: fruit with all tepals and rudimentary androecium attached and position 1 unseparated in all*

Days from anthesis	nl g^{-1} Fwt h^{-1}
	0–1 h
122 ($n=9$)	0.3±0.3
141 ($n=9$)	1.6±0.7
151 ($n=8$)	4.8±2.9

Table 6. *Ethylene production (nl g^{-1} Fwt h^{-1}) by different component parts of the fruit at different stages of ripening (87–120 d unripe, 120–148 d ripening; 150–164 d ripe); ethylene collection periods 0–1 h, except 87 d material (0–7 h)*

	nl g^{-1} Fwt h^{-1}					
	87 d	120 d	148 d	150 d	157–160 d*	164 d**
Single fruit (−tepals) ($n=9$)	0±0	<0.1	3.0±4.6	9.1±8.4	46.1±6.7	106.1±26.2
Tepals ($n=30$)	<0.1	—	—	—	5.5±1.7	—
Rudimentary androecium ($n=42$)	1.4±0.1	—	—	—	6.0±1.2	—
Mesocarp slices	0.2±0.02	2.4±0.2	3.5±0.5	—	47.6	55.5±19.0
Unseparated abscission zone ($n=10$)	—	—	0.1±2.4	—	—	—
Separating abscission zone ($n=65$)	—	—	—	—	7.6	—

* Fruits separating at position 1.
** Fruits already separated at position 1.
Mean fresh weight of tissue parts per separating fruit ($n=35$): intact fruit (−tepals and rudimentary androecium)—11.89 g; the six tepals—136 mg; rudimentary androecium—8 mg; abscission zone—7 mg.

basal mesocarp tissue and not directly with the rate of synthesis at the stigma region (Fig. 4).

Ethylene production as the signal for abscission

(a) *In untreated fruit*: Analyses were made on arrival, of ethylene production and position 1 separation of individual fruits at different stages of shedding from ripe attached fruit to fruit that had shed from the spikelets during the 30 h of transit from the plantation palm to the laboratory bench. The results presented in Fig. 5 show the high levels of production by the fruit already shed during transit (0 h) and the barely detectable production by the fruit that were still firmly attached. Of special interest is the very considerable rise in production over the 9 h from arrival. The initiation of separation at position 1 is closely associated with this rise, as determined at 3, 6 and 9 h.

The highest levels of production are maintained for a relatively short period only (Fig. 5) but as seen from Fig. 4, there is a wave of synthesis that progresses from the apex of the fruit to the base. It seems likely, therefore, that it is the level of ethylene production just above the abscission zone that provides the critical signal for the initiation of the separation events.

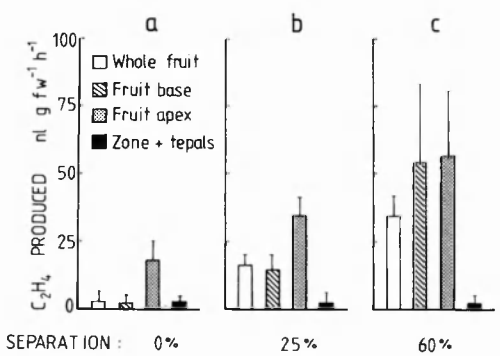

Fig. 4. Ethylene production by different parts of single ripe fruit in relation to percentage separation at position 1. Mean±S.D. ($n=5$). Samples from (a) 148 d; (b) 147 d; (c) 162 d ripe fruit.

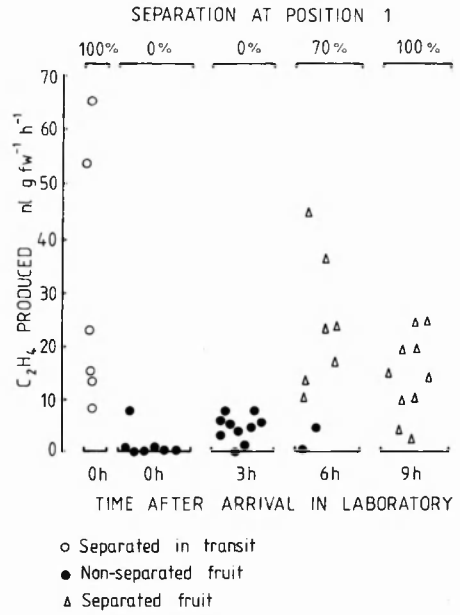

Fig. 5. Rates of ethylene production by single ripe fruit (154 d) in relation to the onset of cell separation at position 1. (○) Separated in transit; (●) non-separated; (△) separated.

(b) *Regulated ethylene production*: If ethylene from the ripe fruit is the signal for abscission at position 1, then the enhancement or suppression of the production should be reflected in the extent of cell separation that follows. When intact fruit (no tepals) are treated with growth regulators for 16 h and ethylene production and subsequent abscission at position 1 are monitored, it is evident (Table 7) that ACC enhances and AOA suppresses both ethylene production and abscission, but, as with other examples of auxin treatments and abscission (Osborne, 1989), auxin blocks the ethylene initiation of the process despite the induction of high levels of ethylene synthesis.

In experiments with tissue slices, ABA was as effective as ACC (or ethylene) in initiating the separation process (Table 3). It is of interest, therefore, that unlike ACC,

Table 7. *Relationship between growth-regulator controlled ethylene production and abscission at position 1 of naked unripe fruit 122 d post-anthesis*

Pedicel fibres inserted in 500 μl H_2O or ACC, 2,4-D or AOA 1 mM for 0–16 h, all fruit then transferred to air. Treatments in triplicate. Experiments using 0.5 mM concentrations of the growth regulators gave lower responses and are not included.

Time (h)	nl g^{-1} Fwt h^{-1}				
	Air	H_2O	ACC	2,4-D	AOA
0–1 h	<0.1	—	—	—	—
17–19 h	<0.1	0.3±0.1	3.1±1.5	6.2±1.9	0.1±0.1
24–25 h	0.2±0.3	0.6±0.3	4.3±1.6	7.7±4.5	<0.1
Abscission at 39 h	0%	40%	100%	0%	0%

ABA does not at once enhance the production of ethylene in treated fruits, but does so in unripe and ripening fruits after a lag period: there is no increase in production by ripe (151 d) fruit. (Table 8). The advancement of abscission can, however, be linked to an enhancement of the ethylene signal in the 141 d fruit, suggesting that although the production by 122 d fruit is increased, it did not (in these experiments) reach the threshold level necessary to initiate the abscission process.

Discussion

Abscission of ripe fruits of the oil palm has been shown already to exhibit unusual characteristics (Henderson and Osborne, 1990; Osborne *et al.*, 1992). The two phase separation, by which the fruit first loosens from the pedicel (position 1) to be followed by a second phase in which the rudimentary androecium becomes separated from the outer rim of the fruit base (position 2) or the rudimentary androecium separates from the inner whorl of tepals (position 3), may be unique to palms. The present paper provides further information on the factors that control these events. It appears that ethylene can initiate (induce), but not accelerate an already induced, first stage of separation at position 1 and that an applied auxin can delay this induction by ethylene, but not suppress an induction that is already expressed (Fig. 2; Tables 3, 7). An ethylene induction can operate in unripe fruit as young as 96 d post-anthesis (Fig. 2), which is before lipid deposition or carotene synthesis has commenced: this corresponds to a stage when ethylene production by the untreated fruit is at only just detectable levels (Table 6). It also shows that zone cells, with competence to separate in response to ethylene, are differentiated before the onset of mesocarp ripening.

Significant ethylene synthesis by the fruit does not occur until the fruit is fully ripe, so ethylene may not be the signal for the initiation and progression of ripening since this can commence as early as 120 d post-anthesis. However, from the time-course of ethylene production by ripe fruit (Fig. 5) during which there is a basipetal progression of synthesis starting from the stigma end (Fig. 4), ethylene appears instead to be a signal for the initiation of separation at position 1. Furthermore, it would seem that in ripe fruit position 1 can perceive and respond to this ethylene signal by separating within 6 h (Fig. 5).

The rudimentary androecium and the tepal bases also show a rise in ethylene production at the same time as the mesocarp, but compared with mesocarp production, which in some fruits can reach over 300 nl g^{-1} Fwt h^{-1}, these attendant parts seldom increase their synthesis to more than 10 nl g^{-1} Fwt h^{-1}. Since the total fresh weight of these parts on a per fruit basis is very small (Table 6) the part they might play in the initiation of abscission at position 1 would seem, therefore, to be quite limited.

Another unusual feature of the second stage of abscission at either the inner or outer surfaces of the rudiment-

Table 8. *Growth regulator enhancement of ethylene production and abscission (at position 1 and 2 or 3) in fruits at different stages of ripeness*

Fruits with tepals and rudimentary androecium intact. Pedicel fibres immersed continuously in 1.0 ml water or 1.0 mM ACC or ABA. Treatments in triplicate. Experiments using 0.5 mM concentrations of the growth regulators gave lower responses and are not included.

Days from anthesis	Time (h)	nl g^{-1} Fwt h^{-1}		
		H_2O	ABA	ACC
122	0–1	0.1	0.7	2.9ab
	4–5	0.8	1.4a	45.8ab
	8–9	1.5	6.0a	33.2ab
	33–34	0.2	12.6a	7.2a
Abscission at 5 d		5%	0%	22%
141	0–1	2.2	1.6	13.1ab
	4–5	9.5	6.5	80.8ab
	8–9	70.6	57.0	195.4ab
	33–34	41.1	143.8a	89.9
Abscission at 5 d		5%	16%	61%
151	0–1	6.9	10.9	11.2
	4–5	23.0	26.8	135.1ab
	8–9	188.0	169.3	218.2a
	33–34	174.6	132.6	109.7
Abscission at 5 d		44%	44%	40%

a = Values significantly different from control value at that time (P = 0.05).

b = Values significantly different from all other values at that time (P = 0.01).

ary androecium or at the tepal bases (positions 2, 3, 4 or 5) is the absence of any cell separation response to either endogenously produced, growth regulator induced (by ACC or ABA) or applied, ethylene. These positions will undergo abscission only after the separation at position 1 has been achieved and only when left in contact with position 1 until the latter has separated. This suggests that some product(s) of separating cells at position 1 are essential components of a signal that passes to, and initiates cell separation in, the outer positions of the abscission zone. Saccharide fragments or other degradation products of middle lamella or cell wall hydrolysis could well be signal molecules emanating from position 1 (Ryan and Farmer, 1991; Fry *et al.*, 1993, and references therein). Also, we do not exclude the possibility that ethylene too, may be an essential component of a complex signal.

In other abscising systems such as the bean, (Tucker *et al.*, 1988), auxin plays the role of a suppressor of gene expressions normally induced during cell separation. The ability of zone cells in oil palm fruit to respond to abscission signals appears also to be suppressed by auxin (Fig. 2; Tables 3, 7). The fact that positions 2 and 3 are bypassed when unripe or ripening fruit from excised spikelets are studied could relate to differences in the endogenous auxin status between the different positional tissue types involved in shedding.

The scheme depicted in Fig. 6 indicates a possible inter-tissue signal transduction control of oil palm fruit abscission.

Although the early signal which initiates the onset of fruit ripening is unknown (but appears *not* to be ethylene) the signal that initiates position 1 cell separation is almost certainly ethylene and this is liberated at a very precise

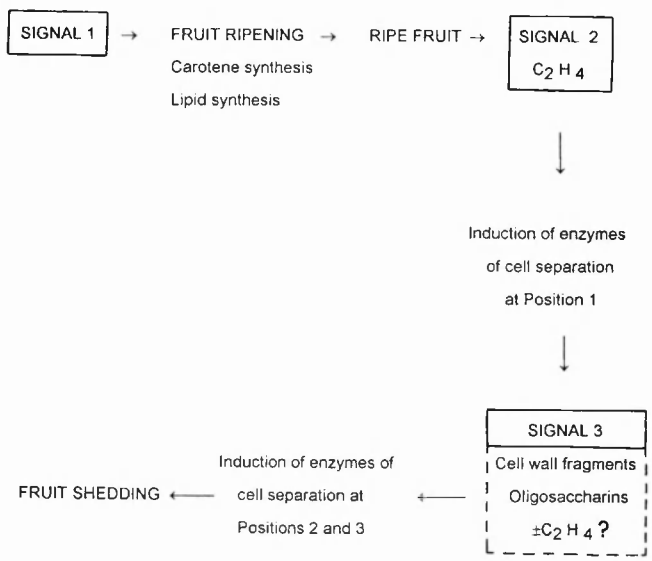

Fig. 6. Proposed sequence of inter-tissue signalling events in the regulation of oil palm fruit abscission.

stage of full fruit ripeness. The cells at position 1 that respond to this signal are, however, positionally differentiated as competent ethylene-responsive target cells even before ripening has commenced. The liberation of ethylene via applied ACC is sufficient to initiate separation of these cells at 120 or 122 d post-anthesis, although the production induced by 1.0 mM ABA after a lag period of 30 h is apparently too low (Table 8). By 141 d (and at 148 d, Table 3) when fruits are already ripening, ABA will enhance abscission. The enhancement at 141 d (Table 8) can be linked to an elevated ethylene formation. No enhancement of ethylene production with either ACC or ABA occurs in fully ripe fruit that is already synthesizing ethylene. This lack of a further rise in ethylene production in response to added growth regulators accords with results published earlier for ripening fruits and senescing leaves in dicotyledons (Roberts and Osborne, 1981)

An additional feature that distinguishes the oil palm fruit abscission from that described for dicotyledonous fruits and leaves (Addicott, 1982; Sexton and Roberts, 1982; Osborne, 1989, and references therein) is the lack of an ethylene-induced endo-β-1,4-glucanhydrolase in certain of the separating positions of the abscission zone, despite the fact that (as in the avocado fruit, Dallman *et al.*, 1989) a highly active enzyme is produced in the mesocarp tissue at full ripeness. Enzymes other than a β-1,4-glucanhydrolase must, therefore, also play a part in the co-ordinated shedding events of the oil palm fruit and this may reflect differences in wall composition between monocotyledons and dicotyledons (Kieliszewski *et al.*, 1990) or between the palms and other monocotyledons (Jarvis *et al.*, 1988), or perhaps, between the different tissue types that make up the abscission zone complex.

A study of the wall cleaving enzymes that are associated with the interrelated cell separations at the several different abscission positions of the ripe fruit and their possible signal products will be the subject of further communications.

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